MOLECULAR CHARACTERIZATION OF MICROBIAL COMMUNITIES AND DEVELOPMENT OF STARTER CULTURE FROM TRADITIONALLY FERMENTED MILK, *AMABERE AMARURANU* IN KISII COUNTY, KENYA.

MOSES BARASA SICHANGI

A Thesis submitted to Graduate School in Partial Fulfilment for the Requirements for the Master of Science Degree in Food Science of Egerton University.

EGERTON UNIVERSITY.

NOVEMBER, 2019

DECLARATION AND RECOMMENDATION

Declaration

I declare that this thesis is my original work and has not been presented in this or any other University for any degree.

Signature: _____ Dated _____

Moses Barasa Sichangi

KM16/14202/15

Recommendation

This thesis has been submitted with our recommendation as University' supervisors.

Signature: _____ Dated: _____

Dr. John M. Nduko, PhD.

Department of Dairy and Food Science and Technology,

Egerton University.

Signature: _____ Dated: _____

Prof. Joseph W. Matofari, PhD.

Department of Dairy and Food Science and Technology,

Egerton University.

COPYRIGHT

DEDICATION

This thesis is dedicated to my dear family; Gertrude Sichangi, Francisca, Sheila, Hillary, Getrude, Doreen, Gloria, Daisy, Joshua, and Emmanuel

ACKNOWLEDGEMENTS

Honour and glory go to the almighty God for His immeasurable love, mercy, care, strength and guidance during my study. I wish to acknowledge Egerton University through the Department of Dairy and Food science and Technology, for their sincere and honest support since I enrolled for my studies. I thank the Michigan State University (Global centre for systems innovation), USA for funding this study. I am grateful to National Commission for Science, Technology and Innovation research permit number NACOSTI/P/19/2492. I'm most indebted to my supervisors Dr. John Masani Nduko and Prof. Joseph W. Matofari for their technical advice, guidance, professional supervision, inspiration and unlimited support during the entire study period. Many thanks also go to the Dean Faculty of Agriculture, Prof. Abdul K. Faraj, the Chairman of the Department of Dairy and Food science and Technology Dr. Patrick Muliro for their support and guidance. Special thanks also go to Mr. Manfred Miheso of KALRO, Njoro for his valuable assistance in the field and molecular samples analysis. Special thanks also go to Mrs. Bernadette Misiko, for her support and guidance in Laboratory analysis. To my fellow students, Francis Irungu, Norbert Wafula, Hillary Indago, Cyprian Syeunda among others, thanks for the good times, your support and for their encouragement hard times. Recognition also goes to my family, St, Augustine chaplaincy Egerton, Christian community members for their encouragement.

ABSTRACT

Spontaneously fermented milks are integral to human diets in many African communities. These products have found their way into the informal market posing a public health concern because their quality and safety is not ascertained. Consequently, there is need to isolate and identify microbial strains with superior fermentative qualities and attributes of health qualities as starter cultures to commercialize production as well as profile potential microbial hazards. The aim of this study was to characterize microorganisms in *amabere amaruranu* for identification of starter cultures for the fermentation of milk under controlled fermentation conditions to improve quality and safety. Forty-six (46) samples of *amabere amaruranu* were collected from Kisii County and characterized for total viable counts (TVC), total coliform counts (TCC), lactic acid bacteria (LAB) and yeasts and moulds using cultural methods. To isolate LAB as potential starter cultures, discrete colonies on MRS agar were selected and purified by repetitive streaking on de Man, Rogosa and Sharpe (MRS) agar. Thirty-seven (37) colonies were selected for phenotypic (Gram staining and catalase test) and physiological characterization DNA of the isolates was extracted for identification by 16S rRNA gene sequencing. The obtained sequences were compared to DNA sequences in the GenBank by the BLASTN Program and aligned in Mega 6.0. Fermentation capability was assessed by lactic acid production and pH change. The bacterial counts were 3.82- 10.98 $\log_{10}$ cfu/ml (TVC), 3.52–9.01 $\log_{10}$ cfu/ml (TCC), and 3.52–11.32 $\log_{10}$ cfu/ml (LAB), while the yeasts and moulds were 0.00–9.05 $\log_{10}$ cfu/ml. Five species of lactic acid isolates were identified by sequencing of which *Lactobacillus plantarum* was the most predominant species. *Lactobacillus plantarum* had good acid production capability within 16 hours. These results show that, it is possible to characterise microbial diversity of *amebere amaruranu* using both cultural and molecular techniques. *Amabere amaruranu* contains diverse microbial populations in terms of type and numbers. *Lactobacillus plantarum* in *amabere* is a potential starter culture that could be used to upgrade production of fermented milk. *Lactobacillus planturum* has good fermentation capabilities hence could be used as starter culture for product development.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
CTAB	Cetyl trimethyl ammonium bromide
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxy Ribonucleic Acid
KEBS	Kenya Bureau of Standards
LAB	Lactic Acid bacteria
MRS	de Man, Rogosa and Sharpe
NCBI	National Center for Biotechnology Research
PCR	Polymerase Chain Reaction
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl Sulphate
TCC	Total coliform counts
TVC	Total Viable count

CHAPTER ONE
INTRODUCTION

1.1 Background information

Most of the communities in Africa have a long history on spontaneous food fermentation that has depended on local knowledge Most of the indigenous food products are informally produced and many still take place at small enterprise level or household level (Elaine and Danilo, 2012). Fermentation is a form of food processing and preserves food and is inherently rooted in village life and traditional cultures (Pederson, 1971; Campbell-Platt, 1994). Fermentation imparts desirable attributes such as taste and aroma. Product toxicity is also decreased while creating product diversity in diets including staple foodstuffs that includes fish, tubers, milk, and cereals. (Chelule *et al*., 2010). Fermentation also decreases bulkiness of materials for easy transportation, enhances nutritive value and appearance of food, and it decreases fuel for cooking (Holzapfel, 2002). Fermented foods reportedly account for one third of all the food consumed in the whole world and 20-40% of the world available food supply.in the current food systems. Fermented contributes a huge component of the global diet to enhance food security and improve livelihoods especially in poor rural areas in developing countries (Chilton *et al*., 2015).

In Africa, a number of traditional fermented products such as milks, breads, cheeses, porridges and alcoholic beverages have been documented (Marshall and Mejia, 2012). Production of these foods relies mostly on indigenous knowledge and they are produced informally at the small-scale level or at household level. Traditional fermentation technology is utilized in Kenya to produce fermented beverages and foods from various raw materials that include bananas, honey, milk, cereals, coconut sap and sugar syrup among others (Nout, 1981; Kunyanga *et al*., 2009; Nduko *et al*., 2017). The common method of preserving milk is fermentation of milk, which many communities in Kenya consume as traditionally fermented milk. The Kalenjin community use *mursik*, the Northern Kenya's pastoralists use fermented camel milk called *suusa*, the Maasai community, call it *kule naoto* and the Abagusii refer to it as *amabere amaruran.*. These communities have been taking these milk products as traditional food products for a long time maybe because of its attributes of flavour, consistency, colour and shelf life (Lore *et al*., 2005; Mathara *et al*., 2008).

Hollowed-out fruit of *Lagenaria* spp is used to make a gourd that is then utilized to prepare fermented milk by spontaneous fermentation. The products are made from either raw milk like *mursik* or boiled milk like *amabere amaruranu*. Plastics and gourds are common types of containers used for fermentation at household level. Gourd-fermented milk is the most preferred by consumers. Back-slopping method is used, which involves addition of the milk to a small portion of previously fermented milk in a container and then left to ferment at ambient temperature (Nduko *et al*., 2017). The potential of back-slopping technology or in this case traditional fermentation technology of producing fermented milk products has not been fully exploited on industrial scale to satisfy the increasing demand for products of traditionally fermented milk by immigrant and urban population. The reasons for this gap include lack of appropriate starter culture that imparts the attributes that these immigrants are used to in the village homes and secondly the production is not under standard hygienic conditions.

Technologically significant species and strains for use in the fermentation industry, which are microbiota in indigenously fermented milk products, are getting lost gradually with new technologies (Nyambane *et al.,* 2014). In making these products, microbes in raw milk and the processing environment serves as natural starter cultures for fermenting the milk. These microbes improve properties that include nutritive value, shelf-life, taste, and texture of milk (Nyambane *et al.*, 2014)

Important and useful microbial cultures for commercial and scientific purposes are identified by isolation and screening of microorganisms from naturally occurring processes. The diverse mixture of lactic acid bacteria and yeasts microorganisms are potentially utilized for innovation of fermented milk products including *amabere amaruranu* and other many fermented milk products (Nyambane *et al.,* 2014). Recent studies identified some lactic acid bacteria including *Lactobacillus plantarum*, *Leuconostoc mesenteroides subsp. Mesenteroides, Streptococcus thermophilus,* yeasts (*Candida famata*, *Trichospora mucoides*), and *Saccharomyces cerevisiae* (Nyambane *et al.,* 2014). Lactic acid bacteria which are probiotics in *amabere amaruranu* in Kisii County have not been characterized comprehensively, especially at molecular level. Food and nutrition security can be achieved by selection of microbial species and strains that

have functional properties for industrial production and for improvement of safety and quality of existing traditionally fermented food products. There is need for isolating these lactic acid bacteria from traditionally fermented milks and characterize them to molecular level so as to package them as starter cultures for the traditionally fermented milks like *amabere amaruranu*. This will enhance product quality unlike using back-slopping and reduce chances of entry of environmental microbes including pathogens when milk is fermented traditionally.

The aim of the study was for characterizing lactic acid bacteria from *amabere amaruranu* as potential starter culture using molecular technique and demonstrate potential for usage as starter cultures

1.2 Statement of the problem

Spontaneous fermentation has been utilized for centuries in production of many African traditional foods including *amabere amaruranu*. Nutritionally, these traditional fermented foods contribute immensely to African diets. However, the scale of production is limited to domestic level and it is not possible to commercialize traditionally fermented milk products because production is not standardized, due to lack of defined standard starter cultures by the communities. This poses a health risk to the consumers. Currently the producers of the product use environmental microbes as starter cultures, which result in various attributes that do not appeal to other consumers, hence low income. Therefore, the purpose of this study was to characterize microorganisms in *amabere amaruranu* for starter culture development to enable production of fermented milk products of high and consistent qualities, which are also safe and high quality and produced efficiently at home or at commercial level.

1.3 Objectives

1.3.1 General objective

To contribute towards food and nutrition security by improving the quality and safety of indigenously processed milk by developing a potential starter culture isolated from traditional fermented milk for production of commercial fermented milk.

1.3.2 Specific objectives

i) To characterize the microbial diversity of *amabere amaruranu* from different sources using cultural and molecular techniques.

ii) To identify potential starter culture from *amabere amaruranu* predominant lactic acid bacteria isolates.

iii) To determine the fermentation capability of lactic acid bacteria isolated from *amabere amaruranu* for use as potential starter culture for product development.

1.4 Hypotheses

i) There is no microbial diversity in *amabere amaruranu* from different sources as characterized by cultural and molecular techniques.

ii) There is no predominant lactic acid bacteria species for the development of a potential starter culture of *amabere amaruranu*.

iii) There is no fermentation capability of lactic acid bacteria isolated from *amabere amaruranu* for use as potential starter culture for product development.

1.5 Justification of the study

To achieve food and nutrition security, traditional foods production especially spontaneously fermented ones, should be commercialized. This can be achieved through isolating and identifying microbial strains with superior natural fermentative qualities and attributes of health benefits that would form a potential starter culture to commercialize the production of *amabere amaruranu* which ensures safety, quality and food security. It was anticipated that this study would contribute towards a body of information about microbial composition of the spontaneously fermented milk of *amabere amaruranu*. According to Akabanda *et al.* (2013), some traditional technologies used for production of fermented foods might reportedly get lost together useful associated microorganisms due to changes in modern socio-economic conditions. Hence, this developed potential starter culture would contribute to preservation of microorganisms associated with spontaneous fermentation. The outcome of this study is a contribution to improvement in health of the people and income of the producers when quality and safety is assured and starter culture is home grown.

1.6 Limitations of the study

This study was carried out as an academic project to fulfill the requirements for author's Master's Degree and most of the experiments that were conducted were based on materials and facilities that were available in the department of Dairy and Food Science and Technology, Egerton

University. The project funding by Michigan State University was not adequate to carry out complete molecular analysis and characterization of all the inherent microorganisms in the *amebere amaruranu* and characterize the full potential of the isolated strains. Therefore, the design of the experiments conducted was time-limited to academic project requirements.

CHAPTER TWO

LITERATURE REVIEW

2.1 Fermented foods

The oldest method of preserving foods that are perishable which is beyond written history by man is the fermentation of foods. The variety of such fermented food products is inherent of different traditions found in the world in different geographical areas where they are processed and cultural preferences (Elaine and Danilo, 2012). The methods of processing were initially not known and occurred by chance, and perpetuated by traditional and cultural values to subsequent generations. For many generations, fermentation method remains an effective means of extending the storage life of milk in Africa (Rahman *et al.*, 2009).

Fermented foods, which may be of either plant or animal origin form an integral portion of the peoples' diet in whole of the world. Fermented food plays a very significant role in the economics and social aspects of developing countries by prolonging the shelf life of foods with aspects which include wholesomeness, acceptability and quality. Every country has their own kinds of fermented food that are represent of the raw materials and staple diet and the available in that region (Lore *et al.*, 2005). The biotechnological method historically developed from the need to preserve food is fermentation. It can be done by bacteria, yeasts, and filamentous fungi, or a combination which hydrolyzes fermentable carbohydrates into metabolic products that include carbon dioxide, organic acids, and alcohols, (Leroy and DeVuyst, 2004). Variety of foods that can be fermented, include cereals, vegetables, milk, fish and meat, (Hutkins, 2006). Lactic acid bacteria (LAB) are of specific interest in fermented foods, due to their ability to lactic acid as a most common metabolite (Stiles and Holzapfel, 1997). This lactic acid prevents of the growth of pathogenic and spoilage microorganisms and thereby extends the shelf life of the fermented foods. Common useful LABs that are associated with foods have been granted the 'generally regarded as safe' (GRAS) status the food grade genera are *Weissella*, *Streptococcus*, *Lactobacillus*, *Tetragenococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, (Wessels *et al.*, 2004). Coagulase-negative *Staphylococci* (CNS) are close to lactic acid bacteria, *Kocuria* spp., and the *Micrococcus* spp. are found naturally as microbiota in fermented meat products close to *Lactobacilli* and *Pediococci* (Leroy *et al.*, 2006). Additionally, in meat, CNS can also be associated with both soft and hard cheeses, as they are

tolerant to salt and acid conditions (Irlinger, 2008). Other bacterial groups that take part in food fermentations include *Propionibacteria*, *Brevibacteria,* and *Corynebacteria,* which are utilized in cheese production, and *Bacillus subtilis* that is utilized for soybean fermentation (Hutkins *et al.,* 2006). Acidifying LAB in environment that is rich of carbohydrates most times grow in close association with yeasts which are often lower in numbers. Yeast species of *Hansenula*, *Saccharomyces*, *Hanseniaspora*, *Candida*, *Torula,* and others, can multiply in these niches causing spontaneous alcoholic fermentation, such as in the spontaneous fermentation method of wine and beer (Fleet, 2007).

2.2 Spontaneously fermented milk

Lactic acid bacteria in the milk hydrolyse the milk sugar lactose into lactic acid that gives the fermented product a specific sour flavour during fermentation. Lactic acid bacteria also synthesize numerous secondary metabolites, organic acids (propionic acid acetic and), hydrogen peroxide and bacteriocins or bactericidal proteins and hydrogen peroxide through distinct fermentation pathways controlled by the genetic material. Bacteriocins are antimicrobial substances that prevent the growth of disease-causing bacteria or pathogens (Abdullah and Osman, 2010). Microbial safety of raw milk is of high concern primarily as the milk is a highly nutritious product that readily supports the proliferation of spoilage microbes (Forouhandeh *et al.,* 2010).

Fermented milk is reportedly much safer than fresh milk because the lactic acid and the bacteriocins destroy coliforms and other disease-causing bacteria such as *E. coli.* Besides improved microbiological safety, frequent consumption of fermented milk offers various health benefits to the consumer, which include reduction in constipation, lowering of cholesterol, protection against diarrhoea, and boosting of the body's immune system (Chung *et al.*, 2010). Spontaneous fermentation of milk results from the actions of numerous species of lactic acid bacteria that proliferate naturally in milk (Sansonnetti, 2008).

2.2.1 Studies on fermented milk outside Kenya

Microbiological characteristics of many fermented milk products are studies that have been conducted in countries including Burkina Faso (Savadogo *et al.,* 2004); Sudan (Abdelgadir *et al.,* 2001); South Africa (Beukes *et al.,* 2001); Zimbabwe (Gadaga *et al.,* 1999); Tanzania (Isono *et al.,*1994); Ethiopia (O'Connor *et al*., 1993); Morocco (Hamama, 1992); Indonesia (Hosono *et al.,* 1989); and Nigeria (Atanda and Ikenebom, 1989) among others. The nature of various

fermented products differs from one region to another. It relies on the local indigenous microflora, which translates into the climatic conditions of the area (Savadogo *et al.*, 2004). Thus, traditionally fermented milk in the areas with a cold climate inherently have mesophilic bacteria that include *Leuconostoc spp.* and *Lactococcus* while the thermophilic bacteria, that include more often *Streptococcus* and *Lactobacillus* occurred in areas with a hot, tropical or subtropical climate condition (Tamine and Robinson, 1988; Kurmann, 1994).

2.2.2 Fermented milk in Kenya

The variety of traditional fermented milk products in Kenya that include mursik taken by Kalenjin community, *suusa* of the North Kenya pastoralists, *amabere amaruranu* of the Abagusii and *Kule naoto* of the Maasai community (Muinde, 2011; Muigei *et al.*, 2013). *Mursik* is reportedly prepared from cows' milk that is fermented in ash-treated gourds. Blood can be added to the fresh milk prior to fermentation, or to milk already fermented. The blood-milk mixture or milk is heated to the boiling point then cooled to an ambient temperature. Then the mixture undergoes spontaneous fermentation which takes three to five days, by the action of the lactic acid bacteria, molds species and yeast (Mathara *et al*., 2008; Muigei *et al*., 2013). Fermentation largely contributes to the health benefits of the rural population rural areas. The preparation of many indigenous or traditional fermented foods and beverages remains a household art to date (Elaine and Danilo, 2012).

2.3 *Amabere amaruranu*

According to Nyambane *et al.* (2014), *amabere amaruranu* is a fermented milk product that is processed spontaneously by fermentation of milk taking place in a gourd container made from the hollowed-out fruit of *Lagenaria* spp. *amabere amaruranu* is most popular amongst the Abagusii, who live in the Kisii county of Kenya. It is usually prepared from cow’s milk that should be heated and cooled then back sloping is used to inoculate the milk which is allowed to ferment at the ambient temperature conditions. Types of containers are utilized for fermentation are plastics and gourd containers. Milk fermented from the gourd is the most preferred. The color of the product is white less viscous, lumpy in nature, and tastes acidic. There is need to carry out more research to have an elaborate information on the microbiological composition of *amabere amaruranu.*

2.4 Starter cultures in fermented milk production

The processes involved in the production of the characteristic flavour and texture of fermented milks are the result of the presence of specific microorganisms and their enzymes in milk. These microorganisms may be bacteria, molds, yeasts or combinations of these (Marshall and Law, 1984; Tamime, 1990). Since these microorganisms initiate or "start" fermentation, they are often referred to as "starter cultures" or "starters". Sanders (1999) defines a starter culture as a microbial strain or mixture of strains, species or genera used to affect a fermentation and bring about functional changes in milk that lead to desirable characteristics in the fermented product.

The most important dairy starter microorganisms are species in the genera of *Lactobacillus, Leuconostoc, Lactococcus* and *Streptococcus*, which composes the lactic acid bacteria (LAB) (Varnam, 1993). According to Varnam (1993), inclusion of the genera *Pediococcus* was proposed. The intestinal microorganism *Bifidobacterium sp*. is also used in starter cultures due to its therapeutic properties (Varnam, 1993). Some of the therapeutic benefits of *Bifidobacterium sp.* include enhancement of the immune system, restoration of the balance of intestinal microflora and anti-carcinogenic activity (Shah, 2001). Yeasts are also included since they are used together with LAB in the production of kefir and koumiss through lactic-alcoholic fermentation (Varnam, 1993).

2.4.1 Functions of starter microorganisms in fermented dairy products

The main role of starters is to carry out fermentation of milk sugar (lactose) to produce lactic acid. Lactic acid is responsible for the distinctive acidic flavour of fermented milks. The acidic conditions produced in fermented milks (pH 4.8 and below) suppress the growth of some spoilage and pathogenic microorganisms (Varnam, 1993). Besides, starters also produce volatile compounds that include acetaldehyde from threonine or sugars and diacetyl from citrate and (Varnam, 1993). These volatile compounds contribute to the flavour/aroma of the fermented products (Varnam, 1993). Citrate in milk is converted by citrate-utilizing LAB like *L. lactis* and *L. mesenteroides* to pyruvate, which is further converted to acetolactate and then diacetyl. In most LAB, such as *L. mesenteroides* subsp. *cremoris L. lactis*, and *L. mesenteroides* subsp. *dextranicum,* acetolactate is enzymatically decarboxylated to acetoin. However, some strains do not possess the enzyme acetolactate decarboxylase, resulting in acetolactate being accumulated that is subsequently oxidized to diacetyl (Hugenholtz *et al.,* 2002). Acetaldehyde is the main flavour compound in yoghurt and is produced by the yoghurt bacteria that include *L. delbrueckii* subsp. *Bulgaricus* and *S. salivarius* subsp. *Thermophilus*.

2.4.2 Classification of LAB starter microorganisms in fermented milks

Several changes have taken place in the taxonomy of bacteria from the time of the publication of the ninth edition of Bergey's manual of systematic bacteriology (Jay, 1992; Sneath *et al.*, 1986). Several of the new taxonomic groups were created after utilizing the developed modern taxonomic methods including rRNA gene sequencing, DNA base composition, DNA homology, cell wall analysis, serological profiles and enzyme profiles, either alone or in combination with traditional methods (Garvie, 1984; Jay, 1992). The LAB group is comprised of at least 8 genera, four new genera, namely *Carnobacterium, Enterococcus, Lactococcus* and *Vagococcus* have been included together with the 4 traditional genera that include *Streptococcus,* Lactobacillus, *Leuconostoc,* and *Pediococcus.* The carnobacteria were previously classified as *Lactobacilli*, while *Enterococcus, Lactococcus* and *Vagococcus* were formerly grouped with the *Streptococci* (Jay, 1992). The LAB group has all its members share the property of producing lactic acid from hexoses (Jay, 1992).

LAB may be divided into either heterofermentative or homofermentative LAB, based on the products of glucose metabolism. Homofermentative LAB produces over 50% of total acid as lactic acid from glucose fermentation. Heterofermentative LAB produces equal molar amounts of lactic acid, ethanol and CO_2 from hexoses (Garvie, 1984; Jay, 1992). All members of the genera *Pediococcus, Streptococcus*, *Lactococcus* and *Vagococcus*, together with some *Lactobacilli* are homofermentative. All *Leuconostocs* and some *Lactobacilli* are heterofermentative (Jay, 1992; Tammie, 1990). Homofermentative LAB possesses the enzymes hexose isomerase and aldolase, but lack phosphoketolase. They use the (EMP) Embden-Meyerhof-Parnas pathway to produce mainly lactic acid from glucose. Heterofermentative LAB possess mainly phosphoketolase enzyme and hence ferment glucose mainly by Hexose Monophosphate (HMP) pathway (Garvie, 1984; Jay, 1992).

The LABs are either mesophilic (optimum growth at 30ºC) or thermophilic (optimum growth at 40–45ºC). *Lactococcus* and *Leuconostoc* spp. are mesophilic, while *L. delbrueckii subsp. bulgaricus* and *S. salivarius* subsp. *thermophilus* are thermophilic (Tamime, 1990). In 1919, the genus *Lactobacillus* was classified by Orla-Jensen into 3 groups viz. *Thermobacterium, Streptobacterium* and *Betabacterium*, based on whether glucose fermentation was homofermentative or heterofermentative and optimum growth temperature.

2.5 Identification and analysis of lactic acid bacteria

2.5.1 Isolation and purification of lactic acid bacteria using culturing techniques

Lactic acid bacteria in food samples are normally determined on M 17 and de Man Rogosa and Sharpe (MRS) agars at the optimal temperatures for 2-3 days (Guetouache *et al*, 2015). These media are supplemented with cycloheximide to prevent the growing of yeasts. The MRS agar plates are anaerobically incubated at 30°C, 35°C and 42°C for three days in order to provide the suitable temperatures for growth *Leuconostoc* spp, mesophilic *Lactobacilli* and thermophilic *Lactobacilli* respectively. For the M17 agar, the plates are aerobically incubated at 30°C for a period of 2 days, in order to achieve the optimal temperature for *Lactococci* (Hussain, S *et al*, 2011). From the plates, randomly selected colonies are streak plated in order to achieve pure colonies of the isolates which are subsequently incubated at 4°C for the plates or at -20°C for the broths that are supplemented with 20% glycerol for further use (Mathara *et al.*, 2004). All isolates are then subjected to Gram staining, catalase and oxidase tests. catalase-and oxidase-negative and Gram-positive isolates are presumed to be lactic acid bacteria and are stored for further analyses. Purification of the isolates is done by repeated pour plating technique using the same agar medium until pure cultures are obtained. Pure cultures are transferred and maintained on MRS agar stabs (Neti and Erlinda, 2011).

2.5.2 Identification of lactic acid bacteria isolates

Lactic acid bacterial isolates can be identified using many tests which include: CO_2 production from glucose, ammonia production from arginine, growth at different pH values, growth at different temperatures (10°C, 15°C, 30°C, 37°C and 45°C), and growth at different NaCl concentrations (Schillinger and Lucke, 1989). Test strains are cultured in duplicates overnight in MRS broth. Catalase production, Gram reaction, and spore formation are the initial tests (Harrigan and McCance, 1976).

Examination of colony characteristics and Cell morphology on MRS agar is followed by separation into phenotypic groups. Only the samples testing positive for Gram stain and catalase test selected for further identification. Growth at different temperatures as observed on MRS broth after incubation for 5 days at 15°C, 37°C and 45°C and Arginine hydrolysis is tested on M16BPC

media (Thomas, 1973). Growth on 4% and 6.5% NaCl is done on MRS broth after 5 days and utilization of citrate is tested on a citrate containing medium (Kempler and Kay,1980).

Acetone production from glucose is assessed using the Voges-Proskauer test (Samelis *et al.,* 1994). MRS-BCP broth medium (BCP 0.17 g/l) is used to perform the biochemical tests. The carbon source is added to the sterile basal medium as a filtered and sterilized solution to a final concentration of 1%. Carbohydrates utilization is assessed after 24^{th} and 48^{th} h of incubation at the optimal temperature. All the strains are tested for fermentation of the following 15 sugars: ribose, L-Arabinose, mannitol, D-xylose, sorbitol, lactose, cellobiose, maltose, trehalose, melibiose, mannose, rhamnose, sucrose, esculine, and D-raffinose. Two drops of sterile liquid paraffin are placed in each tube to ensure anaerobic conditions after inoculation.

Because culturing methods are unreliable for a complete microbial growth and characterization (Jany and Barbier, 2008). Identification of microorganisms by the phenotypic characteristics includes physiological, morphological and biochemical characters (Goodfellow *et al.,* 1985).

Modern trends in microbial systematics including LAB, are geared towards simplifying microbial taxonomy using quantitative methods of analyzing genome similarity (Botina *et al.,* 2006). Lactic acid bacteria, isolated from different sources, including fermented food products belong to the following genera: *Lactobacillus*, *Micrococcus*, *Lactococcus*, *Pediococcus*, *Streptococcus, Staphylococcus* and *Enterococcus*. Numerous researchers have noted the problems encountered when identifying various genera of facultative anaerobic and catalase-negative bacteria. Isolation and identification of species within the genera *Enterococcus, Leuconostoc, Lactococcus, Streptococcus, Pediococcus,* and *Lactobacillus* phenotypically is particular difficult for microbiologists (Facklam *et al.,* 1989). *Enterococcus faecalis, S. thermophilus*, *Enterococcus faecium,* and *Enterococcus durans* are even harder to distinguish using phenotypic methods because they have similar phenotypes. Strains with characteristics similar to *Enterococci* and *Streptococci,* are also common and similarly hard to differentiate. As such, these strains have been are poorly identified in the past (Stepanenko, 1999).

Identification of lactic acid *Lactobacilli* using phenotypic methods is also tedious and difficult. For instance, identification of L. *delbrueckii* subsp. *rhamnosus, L. delbrueckii* subsp. *delbrueckii, L. delbrueckii* subsp. *lactis, L. bulgaricus, L. plantarum, L. paracasei* and *L. casei,* on the basis of their physiological and biochemical properties is hard since they share many

phenotypic characteristics. In another example, Nyambane *et al*. (2014) were unable to identify 40% of the yeast isolates using the biochemical Analytical Profile Index (API) system. Therefore, for precise species attribution of microorganisms, modern methods based on gene sequencing, polymerase chain reaction and typing methodologies need to be used (Dellaglio *et al.,* 2005).

2.6 Molecular diagnostic methods of lactic acid bacteria

The use of nucleic acid–based tools has become more frequently thanks to high throughput potential arising from PCR amplification or *ex situ* or *in situ* hybridization with RNA, DNA, or peptide nucleic acid probes. Notably, 16S rRNA has been used to link diagnostics and phylogenetics and the ability to query a sequence to databases provide unlimited opportunities (Botina *et al.,* 2006). 16S rRNA gene–based methods are superior and robust compared to traditional phenotypic approaches, which are have low resolution and are unreliable when applied to analysis of compositions and activities of bacterial communities and populations.

Apart from rDNA sequencing an extensive collection of other DNAs based sequencing methodologies have been innovated and are frequently applied to different groups of bacteria (Amor *et al*., 2007). These methods are able to analyze individual micro and macromolecules or whole cells. This ability to analyze whole cells offers the opportunity for analysis of physiological properties in intact cells *in situ* using substrates or fluorescently labeled probes in coupled with high-throughput technologies like flow cytometry. These systems are of notable usefulness in providing information on the viability and stresses in lactic acid bacterial cultures (Amor *et al*., 2007).

2.6.1 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was developed for the identification of point mutations in the 1980s and was first used to analyze microbial communities at the beginning of 1990s (Muyzer *et al.,* 1993). This technique uses electrophoresis in a gradient of a denaturant to separate DNA fragments of the same length but of different molecular weight. Temperature Gradient Gel Electrophoresis (TGGE) uses a similar principle of separation but uses the temperature gradient and the chemical compounds in the gel as denaturants. The DNA fragments are pushed through the denaturing gradient by a constant electric field.

In order to prevent complete denaturation of the fragments by melting is prevented by a high temperature stable GC-rich sequence that does not melt known as the GC clamp. This GC clamp consists of 20–40 bases and is routinely attached to the 5'-end the primers used in PCR to

make them thermostable. The sequence 5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3' is the most frequently used GC clamp (Muyzer *et al*., 1993). The choice of the GC clamp is dependent on melting properties and suitability at specific conditions (Muyzer *et al.*, 2004).

Results of DGGE analysis are displayed as an array of bands of different intensities and the intensities correspond to frequency of PCR amplicons in the mixture. Importantly, if the selected bands are cut and directly amplified and sequenced, they often can result in bands of identical taxonomy. In some cases, such as when dealing with diverse communities, cloning is required before sequencing because the bands are often multiple or contain background DNA. Alternatively, the gels are hybridized taxon, genera, species or strain specific probes for identification of the bands. Additionally, DGGE analysis has also been successfully used analyze functional gene populations (Wartiainen *et al*., 2008; Sakurai *et al.*, 2007; Gremion *et al.*, 2004).

Separation of 16S rRNA gene PCR-amplified segments of different sequences by DGGE is comprehensive and unique tool for application to characterization of bacteria. Downstream identification of groups can be achieved through cloning and sequencing of the cut bands or by binding of the genetic profile (Muyzer *et al*., 1993). Successful application of PCR-DGGE was in monitoring the development of LAB population for production and ripening of artisanal Sicilian cheese (Randazzo *et al*., 2002). Thus, PCR-DGGE has proven to be a worthy alternative tool that can be applied to enable rapid detection and identification of various LABs in food and food products.

2.6.2 Sequence-based techniques for bacteria

DNA sequencing explores variations within sequences of house-keeping genes to identify bacterial species. This is because sequence variations in this region do not occur frequently because of conserved nature of genomes. As such, this method is an alternative to the conventional techniques. The most commonly targeted house-keeping genes exploited in bacterial identification include *gyrB,* 16S rRNA, *rpoA, rpoB, rpoC, rpoD* among others (Glazunova *et al*., 2009). This technology is so sensitive that it can detect micro-heterogeneity within a species arising from changes in a few base pairs of the sequences and group genotypes into variant genotypes such as sequevars and subspecies (Clarridge, 2004). Thus, gene sequencing has the power to discriminate microbial strains more precisely than conventional techniques. When sequence data is subjected to homology search, 80% match shows a similarity within a described species and 10% represent

a new species within a genus while the remaining 10% may denote novel taxa. This is only made possible through sequence-based identification techniques (Drancourt *et al*., 2000). The procedures followed during sequence-based identification of are the same for all organisms (see Figure 1).

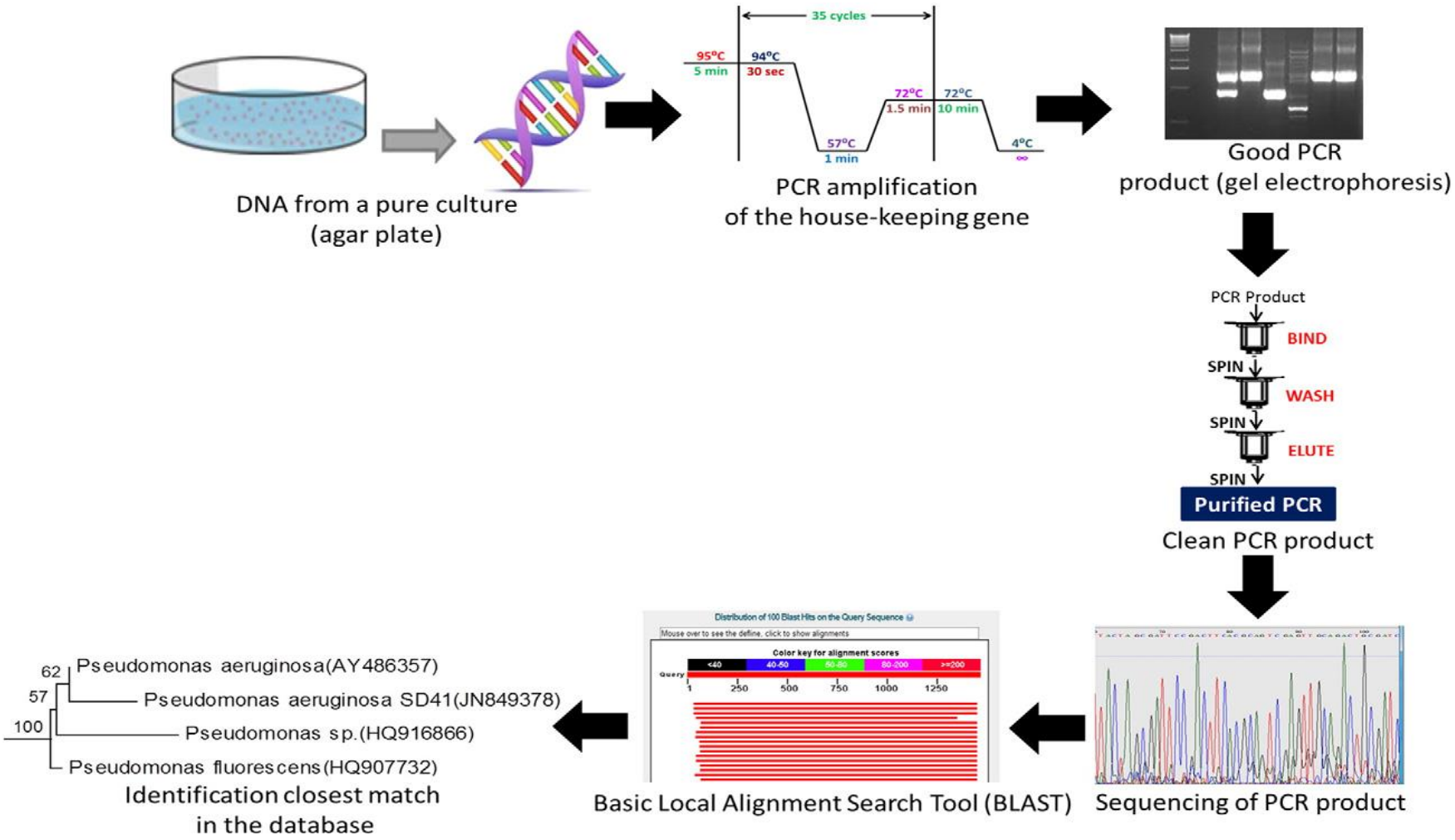


Figure 1. Flow diagram showing the steps involved in bacterial identification using housekeeping sequencing approach Adopted from Surajit *et al.* (2014)

Because there are numerous types of bacteria in the environment, techniques used to identify them are complex and confusing (Spratt, 2004). The use of 16S rRNA gene nucleotide sequence has however, been trusted as the most suitable method for microbial identification and phylogeny.

The 16S rRNA gene has gained infamy as the method of choice for microbial identification because of many reasons. First, the 16S rRNA gene occurs in all organisms and performs the same function. Secondly, it is a conserved the gene sequence. Thirdly, the gene is of desirable size at about 1500 bp which is easy to sequence while at the same time large enough to contain sufficient information for identification and phylogenetic analysis (Clarridge, 2004). This technique gained popularity after sufficient submission of the 16S rRNA sequences in the database and because of easy access to suitable gene amplification primers Comparative sequencing and analysis of this gene has both advantages and disadvantages. The main advantage is the accurate and rapid identification. This is because unlike in conventional techniques such as gas chromatography and mass spectroscopy that require specified equipment and expertise, sequencing identification is mostly automated (Clarridge, 2004). For slow growing bacteria, conventional identification is tedious because the microbes take a lot of time to grow. 16S rRNA sequencing is a unique technology because the results are produced in a considerably shorter time. This technique can also be used to identify isolates which exhibit characteristics that are morphologically atypical (Gee *et al*., 2004). Combining the phenotypic tests, 16S rRNA sequencing, and DNA–DNA hybridization tests has resulted in discovery of new bacterial species and genera (Staley, 2006).

Sequencing of the 16S rRNA gene has been utilized for identification of microorganisms in fermented foods. For instance, Satish *et al*. (2011) isolated more than twenty strains of lactic acid bacteria in traditional fermented foods like *Mor Kuzhambu, Kallappam batter* and *Koozh* in South India. Furthermore, the researchers used antimicrobial activity, morphological, biochemical and physiological characteristics to select 6 strains. Identification by molecular technigues revealed the presence of *Lactobacillus plantarum, Weissella paramesenteroides* and *Lactobacillus fermentum* were identified through amplification and sequencing of 16S rRNA gene and sequence homology of the isolates. Among the 6 isolated strains *Lactobacillus plantarum* AS1 showed the highest antimicrobial activity and was hence chosen as a bio-preservant of cheese and other effective probiotic applications.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study sites

The study was conducted in Kisii County, (Figure 2) South-Western Kenya, in Nyanza region, on Latitude: 0° 41' 0 N and Longitude: 34° 46' 0 E. Kisii highlands had a humid climate and good soils with a high agricultural potential. The local rainfall averages 1500 mm per year, there were no pronounced wet and dry seasons, dry season occur from December to February and a rainy season from March to May and short rains from October to November. Average daily temperature in the study area was approximately 14.8^{o}C (Kisii County Government, 2019)

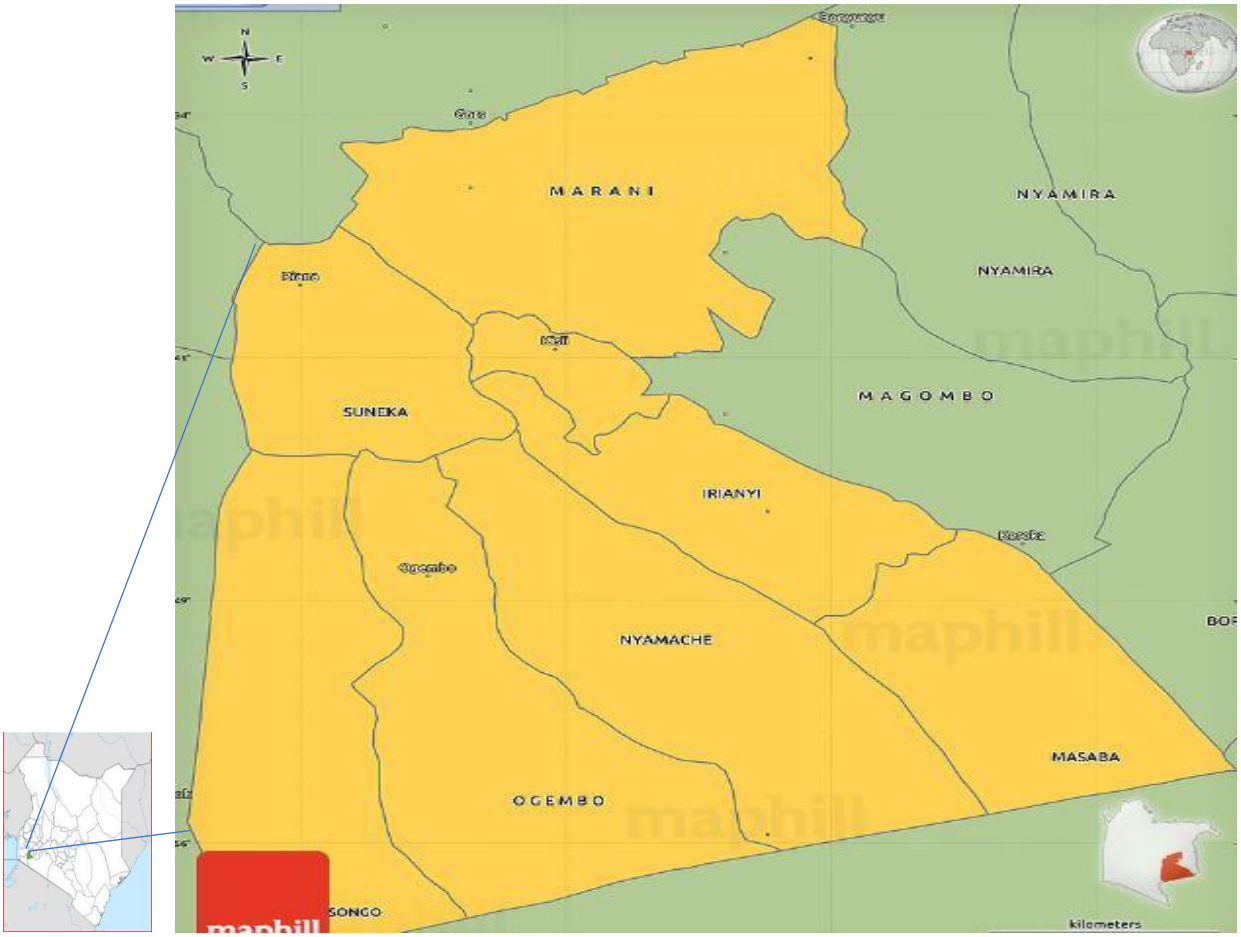


Figure 2: Map showing the source of samples

Source: (www.maphill.com, accessed 25th Sept., 2019)

The research study was conducted at the Microbiology laboratory in the department of Dairy and Food Science and Technology, Egerton University. Molecular work was done at the KALRO, Njoro. Sequencing was done at Inqaba Biotech Industries in South Africa.

3.2 Experimental Design

A quasi experimental research design (not randomized) was used in this study because random assignment was not relevant. Purposive sampling was employed to target households and commercial milk vendors where *amebere amaruranu* was processed after conducting a reconnaissance. Sample was *amebere amaruranu* collected from different households and the milk vendors/processors Sources of *amebere amaruranu* were eight households who were willing to provide the milk and thirty-eight commercial milk producers/vendors that were purposively selected. Samples were obtained in triplicates from each household or milk vendors. The different milk sources (households and vendors) were independent variables while type and microbial counts (TVC, TCC, yeast and molds and LABs) were done to tabulate their levels and show microbial diversity (dependent variables).

3.2. Conceptual framework

Conceptual framework showing the relationship between independent variables, intervening factors and the dependent variables of the experiment is shown in Figure 3.

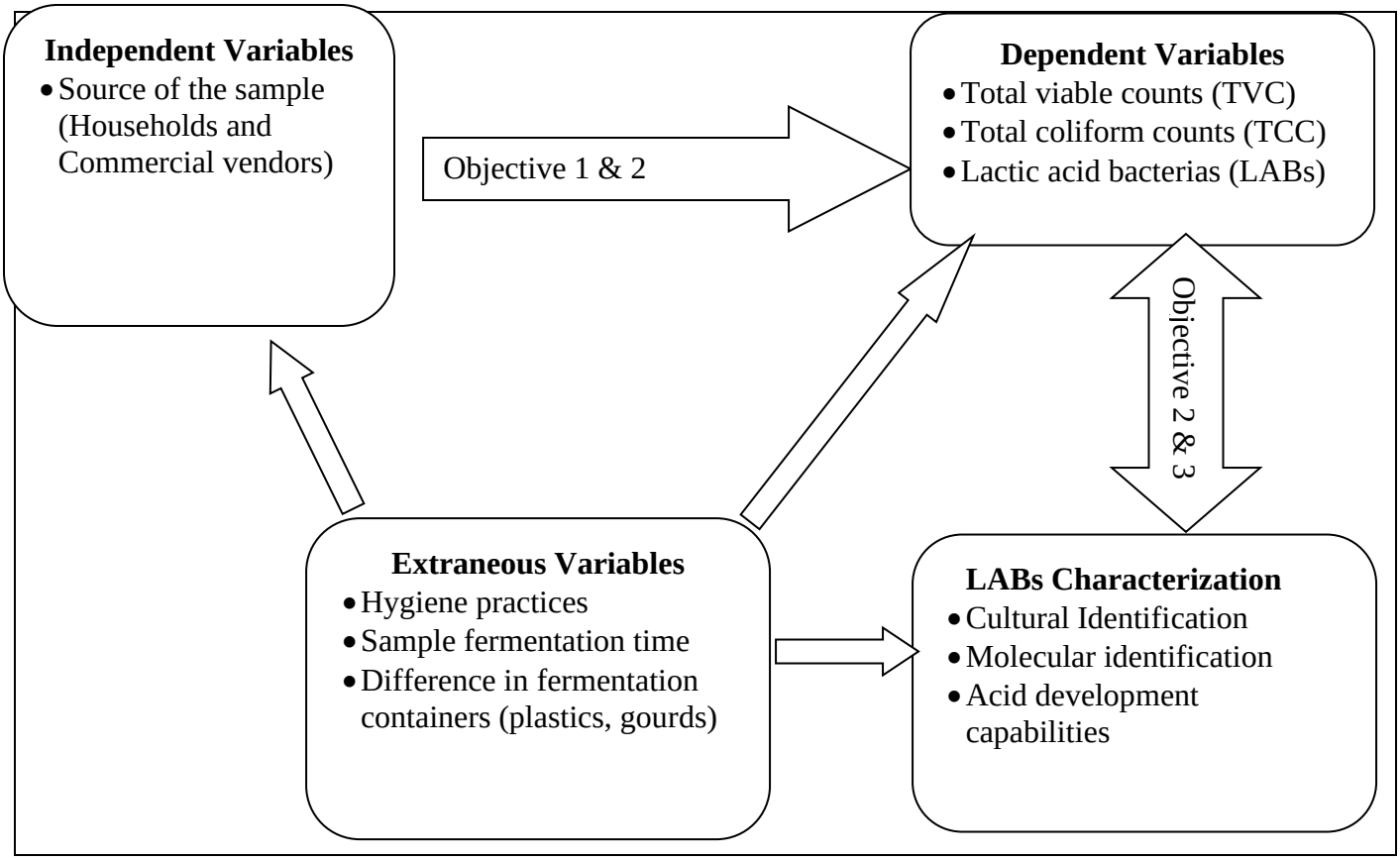


Figure 3. Conceptual framework based on project objectives.

3.3 Sample collection

Amabere amaruranu samples were collected from purposively selected households and commercial vendors/processors. Commercial vendors/processors were the individuals who were selling *amabere amaruranu* to consumers at retail level in shops. At least 200 ml of sample was obtained from each farmer/ vendor during sampling. Three replicates were drawn from the same sample for analysis after thorough mixing.. Sterile (sterilized using the autoclave at 121˚C for 15 min) plastic containers were used to pick the sample, where the farmer was requested to provide the milk(amabere amaruranu) in the container in which they fermented the milk spontaneously. The milk sample was then shaken well to obtain homogeneity and sample drawn. Samples were

drawn asceptically by pouring into the sterile labelled containers and sealed air-tight then transported in a cool box at 4˚C to Egerton University for analysis within 3-4 h. The samples were analysed immediately for microbial characteristics after reaching the laboratory.

3.4 Enumeration of microorganisms

Different microorganisms were enumerated including the total viable counts, lactic acid bacteria and yeast and moulds. They were analysed as shown below.

3.4.1. Enumeration of total viable counts (TVC).

Sterile diluent (90 ml) [1% peptone (Difco, Detroit, Michigan, USA), 0.85% NaCl, pH 7.0] was used to homogenize 10 ml sample using astomacher (Stomacher- Bagmixer, Buch and Holm). The same diluent was also used to make ten-fold serial dilutions from 10^{-1} to 10^{-9} . Aliquot (0.1 ml) of each appropriate dilutions was spreadplated while 1 ml was pour-plated in duplicates on various media for enumeration of isolates. Aerobic mesophilic bacteria were counted by the pour plating technique using Plate Count Agar (PCA) (Oxoid Ltd, Basingstoke, Hampshire, England) and incubated at 30^{o}C for 48 -72 h.

3.4.2, Enumeration of total Coliform Counts (TCC).

Enterobacteriaceae were enumerated through pour plating technique using Violet Red Bile Agar (VRBA) (Oxoid Ltd, Basingstoke, Hampshire, England), incubated at 37^{o} C for 24 h. They were counted and recorded in three replicates.

3.4.3. Enumeration of yeast & moulds

Yeasts and moulds were enumerated by pour-plating technique using Potato Dextrose Agar (PDA) (Oxoid Ltd, Basingstoke, Hampshire, England), pH 5.6±0.2 with chloramphenicol (100 mg/l) (Oxoid) added and incubated at 25^{o}C for 3-5 days.

3.5 Enumeration, isolation and characterizatiobn of lactic acid bacteria

3.5.1 Enumeration lactic acid bacteria

Lactic acid bacteria were enumerated by the pour-plating technique using de Man, Rogosa and Sharpe (MRS) agar (Oxoid Ltd, Basingstoke, Hampshire, England) incubated at 35^{o}C for 2 days using the Anaerocult A pack (Merck, Darmstadt, Germany).

3.5.2 Isolation of lactic acid bacteria

Discrete colonies selected from from pour-plates with highest dilution of MRS agar were isolated based on their size, shape, gloss, and colour using a sterilized innoculation loop. The isolated colonies were purified by streaking thrice on isolation media MRS using a sterilized

innoculation loop and stored in 0.25 mol/L sucrose solution at -18°C for successive identification tests.

3.5.3 Phenotypic characterization of lactic acid bacteria

Gram reaction and catalase tests were carried out to confirm the primary characteristics of lactic acid bacteria to identify whether they were gram positive and catalase negative. Colonies on individual culture plates were described based on their morphological characteristics such as shape, size, elevation, surface characteristics, and edges. Grams stain was used for bacterial cell differentiation into rod-shaped (bacillus) or round-shaped (coccus). Lactic acid bacteria are stained blue/purple colour which is Gram positive but pink/red if Gram negative after viewing using a light microscope.

Catalase test: The organisms to be tested were put in sterile slides. A drop of 3% hydrogen peroxide (H_2O_2) was added to the colony and emulsified. Production of gas immediately or after 5 minutes indicated a positive result.

3.7 Physiological characterization of lactic acid bacteria

The isolated pure culture isolates were further screened for identification based on physiological tests of ability to grow at some salt concentrations and temperatures. All the isolates that were catalase negative and Gram positive were the ones selected for these tests. The isolates were cultured on MRS broth and turbidity after 72 h of incubation was observed visually in order to determine ability to growth at 15^{o}C and 45^{o}C. Gas production resulting from glucose metabolism was assesed on MRS broth basal medium according to the method previously described by Harrigan (1998). Determination of salt tolerance was carried out in MRS broth laced with 4% (w/v) 6.5% NaCl prior to inoculation and incubated for 4 days at 37^{o}C. Increase in the turbidity of the solution was detected by visual inspection and indicated a positive result.

3.8 Molecular characterization of lactic acid bacteria

3.8.1Genomic DNA Isolation

Genomic DNA was prepared using the procedure of Cardinal *et al.* (1997). Briefly, 10 ml of (12 hour- old grown in MRS broths was used for DNA extraction. Cells were harvested in a microcentrifuge **(**Eppendorf benchtop centrifuge, model 5804) and suspended in 200 µl 1xTE

buffer (pH 8) containing 30 mg/ml lysozyme, 25% sucrose. The samples were incubated for 1 h at 37ºC and 370 µl, 1x TE containing 30 µl, of 10% sodium dodecyl sulphate (SDS) and 1mg/ml proteinase K were added. The cell suspensions were incubated for 1 h at 37 °C and cell lysis was done by adding 80 µl CTAB/NaCl solution (10% cetytrimethyl ammonium bromide, 0.7 M NaCl) and 100 µl 5M NaCl. The samples were incubated for 10 min at 65°C to allow for heat lysis. Chloroform isoamyl purification was performed twice using equal volumes of chloroform (chloroform/isoamyl alcohol: 24/1). Equal volume of chloroform/isoamyl alcohol was added and followed by centrifugation for 5 min at 4000*g* (which is equivalent to 6000 rpms of the centrifuge) and the aqueous phase carefully transferred into a new microfuge tube. This process was repeated.

Genomic DNA precipitation was done by addition of 500 µl of isopropanol and the precipitated DNA was washed in 500 µl of 70% ethanol. DNA was pelleted from 70% ethanol after washing by centrifugation for 10 min at 4000*g* Ethanol was poured off and the pellets were airdried at room temperature for 10 min. Dried pellets were resuspended in in 100 µl 1xTE containing 100 µg/ml RNase and incubated for 1h at 37°C. DNA was dissolved by two cycles of heating at in a water bath 80°C for 10 min followed by cold shocking at -20°C for 20 min.

The dissolved DNA was tested for quantity and quality using nanodrop photometer and gel electrophoresis. The gel electrophoresis was done as follows; the extracted DNA was resolved on a 0.8% agarose gel previously stained with 0.3µl ethidium bromide at 100V constant voltage for 30 min visualised using a UV transilluminator, model MGU-502T, C.B.S. Scientific, U.S.A. The resultant bands were scored for their presence, brightness and sharpness against the commercial standard Lambda DNA Ladder. For nanodrop spectrophotometry, 1 µl sample was loaded to the nanodrop pedestal and read after blanking with ultrapure molecular grade water. Concentration and purity of the DNA was thus determined this way.

Genomic DNA samples were dissolved and then stored at -20°C before they were sent to Inqaba Biotech industries Ltd, Pretoria, South Africa, for sequencing using group-specific primer pairs Lac1 (5'AGCAGTAGGGAATCTTCCA') and Lac2-GC (5'GATTYCACCGCTACACATG'3) to detect the genera *Leuconostoc, Lactobacillus, Weissella* and *Pediococcus* (Walter *et al*., 2001) and ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATAT GC-3') for discrimination of fungal species based on the internal transcribed sequence (ITS) (5.8S rDNA-ITS regions of the fungal isolates) (Kurtzman and Robnett, 1998). The 16S rRNA gene-targeted primers pair 907R(C)

(5'CCGTCAATTCCTTT(AG)AGTTT3') and 1492R (5'GG(CT)TACCTTGTTACGACTT3') were used to analyze sequences that were undetected using Lac1 and Lac 2 and ITS1 and ITS 4 primer pairs.

3.8.2 Sequence data analysis

The partial rDNA nucleotide sequences were analyzed and determined using the BLAST algorithm (http:// www.ncbi.nlm.nih.gov/blast; Altschul *et al*., 1997). Consensus sequences were imported into MEGA version 6.0 software (http://www.megasoftware.net; Tamura *et al*., 2007), with which a sequence alignment and phylogenetic trees were created based on the neighbor-joining (NJ) method. This method was chosen because of its ability to give higher iterations and because it is flexible enough to allow bootstrapping, this enabled precise selection of parameters and hence increased reliability of results. The percentage of bootstrap confidence levels for internal branches, as defined by the MEGA program, was calculated from 100 random re-samplings.

3.9 Technological characterization of isolates as potential starter cultures for product development.

3.9.1. Acidifying activity of the isolates

Titrimetric and potentiometric (pH measurement) methods were applied in order to determine acidifying activity of the isolates (Sarantinopoulos *et al.*, 2001). The isolates were thawed from frozen stocks in MRS broth at 37°C for 24 h. Duplicate inoculations of 0.1 ml of overnight grown cultures were prepared in sterile UHT skim milk broths (10 ml). After the 3^{th}, 5^{th}, 8^{th} and 16^{th} h incubations at 24°C, 37°C and 45°C, of aseptically transferred 2 ml aliquots were used for the procedures for determination of acidity development and pH change. Then graphs were plotted for comparison for the different isolates. Potentiometric method was used to determine pH of the samples using a pH meter with glass electrode (Hanna instruments, PH 211 Microprocessor PH Meter) was used. Before using, pH meter was calibrated with buffer 1 (pH 7.0) and buffer 2 (pH 4.0). The glass electrode was dipped into the samples after the calibration and the pH of each sample was recorded.

To monitor production of lactic acid, 2 drops of phenolphthalein were used as an indicator and to 9 ml of the sample aliquots and standardized 0.1 N NaOH solutions were used to titrate the samples. Upon observing the pink colour, titration was terminated and the volume of the 0.1 N NaOH recorded and 1 ml of 0.1 N NaOH was equated to 9.008 mg of lactic acid. The results were

eventually presented in mg/ml. Standardized 1N HCl was used to standardize sodium hydroxide after standardization with sodium bicarbonate as follows. Sodium bicarbonate (0.4g, 0.3g and 0.2 g) was dissolved in 100 ml deionized water and titrated using 1-2 drops of phenyl orange indicator. When the pink colour appeared, titration was terminated and HCl consumed was recorded as 8.6 ml (m3=0.3g), 4.6 ml (m1=0.2g) and 6.6 ml (m2=0.3g). The samples were then boiled for 1-2 s to remove CO_2 and left to cool. If the samples maintained a steady colour, the volume of HCl consumed was recorded as the titration volume but if the colour of the samples disappeared the process of titration was continued until a steady colour was achieved. At the end, factors of consumed HCl were calculated as shown by the formula.

T = m x S x F x N F1HCl = 0.8647 F2HCl = 0.9335 F mean HCl = 0.9042 F3HCl = 0.9144

T =titre in ml, m = mean of HCl, S = sample weight, N = Normality of NaOH, F = Factor values of HCl.

Factor values of NaOH were deciphered by titration of a standardized solution of 1 N HCl using phenol phenolphthalein. Exactly 50 ml and 100 ml of 1 N HCl (F=0.9042) were used to achieve this end. After addition of the indicator, 0.1 N NaOH added to 1 N HCl. When the colour changed from colourless to pink, the 0.1 N NaOH were recorded as 5ml since V1=50 ml and 9.5 ml because V2=100ml. as such the factor value of 0.1 N NaOH was obtained using the equation:

F mean HCl x N1 xV1 = N2 xV2 F1NaOH= 0.9042 F mean NaOH = 0.8816

F2 NaOH= 0.8589

In evaluating the final results, first, the results of isolates (pH and lactic acid mg/ml values converted into percentage by dividing the titre with 10 corresponded to 0, 3, 6, 9, 24 h). Lactic acid production was plotted against time and pH and time graphs were drawn in Microsoft Excel programme.

3.10 Statistical data analysis

Quantitative data obtained was analysed using SAS software version 9.1. Analysis of variance (ANOVA) was used to test the hypothesis at 95% confidence level. Means were separated Tukeys' HSD (Honestly Significant Difference). The following statistical model was used in the analysis of data:

$Y_{ijl}=\mu + \tau_i + \varepsilon_{ij}$

where: $Y_{ijl;}$ observation made on the response variable; μ; overall mean; τ_i; i^{th} effect of the *amebere amaruranu* source and $\varepsilon_{ij;}$ random error.

CHAPTER FOUR

RESULTS

4.1 Characterizing microbial diversity of *amabere amaruranu* from commercial and households.

The log_{10} means ± stderr of microbial counts of the various microorganisms are presented in Table 1. For TVC, the mean counts were 10.20± 0.10 log_{10} cfu/ml for commercial producers while it was 7.02 ± 0.08 for the household samples and it ranged between 3.82 and 10.98 log_{10} cfu/ml (Appendix 1). The mean TCC count was 7.60 ± 0.20 log_{10} cfu/ml for commercial producers and 6.57 ± 0.09 log_{10} cfu/ml for the household samples and the ranges were between 3.52 and 9.01 log_{10} cfu/ml (Appendix 1). On the other hand, the mean LAB for commercial producers was 10.66 ± 0.11 log_{10} cfu/ml while it was 6.84 ± 0.10 log_{10} cfu/ml for household samples and the lowest value recorded for LAB was 3.52 log_{10} cfu/ml while the highest was 11.32 log_{10} cfu/ml (Appendix 1). The yeasts and moulds were also present in most samples with mean values of 8.09± 0.08 log_{10} cfu/ml for commercial producers and 6.20 ± 0.17 log_{10} cfu/ml for households while the highest counts recorded was 9.05 log_{10} cfu/ml while they were absent in some samples (Appendix 1). For all the microorganisms, commercial sample's mean microbial counts were significantly higher ($p<0.05$) than the counts of samples from households.

Table 1. Means of various types of microorganisms in samples of *amebere amaruranu* obtained from commercial processors and households in Kisii.

Sample source	TVC	TCC	LAB	Yeasts and Molds
Commercial milk producers	10.20^{b} ± 0.10	7.60^{b} ± 0.20	10.66^{b}± 0.11	8.09^{b}± 0.08
Households	7.02^{a} ± 0.08	6.57^{a} ± 0.09	6.84^{a} ± 0.10	6.20^{a} ± 0.17

Key: TVC= Total Viable Count; TCC = Total coliform Count; LAB = Lactic Acid Bacteria and Stderr = Standard error.The means ±Stderr are averages of triplicate measurements. Means followed by different superscript letters within the column are significantly different ($p\leq 0.05$).

4.2 Characterization of Lactic acid bacteria isolates

All the isolates obtained on MRS agar were catalase negative and Gram positive (Table 2). For growth characteristics, at 15°C, 34 isolates were growing while three isolates did not grow. This was in contrast to growth characteristics at 45°C where 27 of the 37 isolates grew at this

elevated temperature. With regard to growth characteristics on medium containing NaCl, over 80% of isolates grew at 4% NaCl while only 70% of the isolates grew on the media containing 6.5% NaCl. Among the isolates, 81% of the isolates were rods, 13.5 appeared as short rods, while 5.5% appeared as cocci as tabulated (Table 2).

Table 2: The growth of Lactic acid bacteria culture isolated from MRS media, Gram staining, catalase reaction and growth at different temperatures and salt concentration.

Number	Isolate code	Gram Reaction	Catalase reaction	Growth at 15°C	Growth at 45ºC	Growth at 4% NaCl	Growth at 6.5% NaCl	Cell shape
1	D_2C	+	-	+	-	/+	+/-	Short rods
2	DW	+	-	+	-	+	-	Short rods
3	R_1^3SC	+	-	+	+	+	-	Rods
4	R_1^3SW	+	-	+	-	-	+	Rods
5	R_2^3C	+	-	-	-	+	+	Rods
6	R_2^3SRW	+	-	+	+	-	-	Rods
7	M_1^1W	+	-	+	-	+	+/-	Short rods
8	M_2^1W	+	-	+	-	+	-	Short rods
9	M_2^2WC	+	-	+	-	+/-	-	Short rods
10	M_3^1DW	+	-	+	-	+	-	Rods
11	M_3^1SC	+	-	+	+	+	-	Rods
12	B_1^6SW	+	-	+	-	+	-	Rods
13	B_2^6SW	+		+	-	-	-	Rods
14	B_2^6DW	+	-	+	-	-	+	Rods
15	B_1^4CH	+	-	+	+	-	+	Streptococci
16	B_1^4SW	+	-	+	-	+	-	Rods
17	B_1^3SW	+	-	+	-	+	-	Cocci

Number	Isolate code	Gram Reaction	Catalase reaction	Growth at 15°C	Growth at 45ºC	Growth at 4% NaCl	Growth at 6.5% NaCl	Cell shape
18	$A4^{5}W$	+	-	+	-	+	+	Rods
19	$A22^{5}C$	+	-	+	-	+	+	Rods
20	$A17^{6}C$	+	-	+	-	+	+	Rods
21	$A14^{6}W$	+	-	+	-	+	+	Rods
22	$A9^{6}W$	+	-	+	-	+	+/-	Rods
23	$A21^{5}W$	+	-	+	-	+	+	Rods
24	$A21^{5}C$	+	-	+	-	+	+	Rods
25	$A10^{6}C$	+	-	+	+	+	+	Rods
26	$A18^{6}W$	+	-	+	-	+	+/-	Rods
27	$A15^{6}C$	+	-	+	-	+	+	Rods
28	$A13^{6}W$	+	-	+	+	+	+	Rods
29	$A15^{6}W$	+	-	+	+	-	+	Rods
30	$A9^{4}W$	+	-	+	-	+	+	Rods
31	$A12^{5}W$	+	-	-	-	+	+	Rods
32	$A10^{6}W$	+	-	+	-	+	+	Rods
33	$A21^{6}W$	+	-	-	-	+	+	Rods
34	$A17^{6}W$	+	-	+	-	+	+	Rods
35	$A2^{6}W$	+	-	+	+	+	+	Rods
36	$A3^{5}W$	+	-	+	+	+	+	Rods
37	$A27^{6}W$	+	-	+	+	+	+	Rods

4.3 Molecular characterization of Lactic acid bacteria

4.3.1 DNA isolation and quantitation

The genomic DNA extracted from the isolates produced intact bands as shown in the image (Fig. 4 upon electrophoresis, indicating that gDNA was successfully extracted. For gel electrophoresis all the samples showed bands of same clarity and sharpness as the lambda DNA ladder showing that they were of good quality and integrity.

.

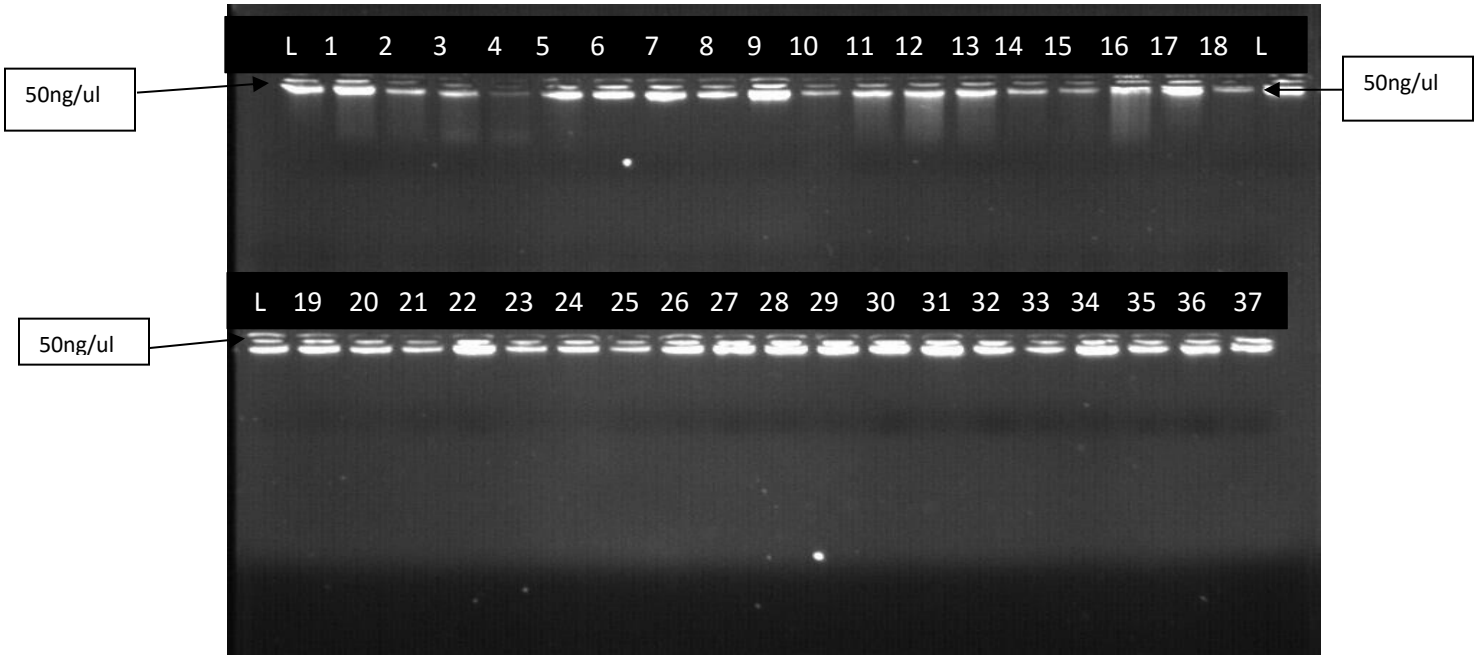


Figure 4: DNA quantification gel image;

Key: L; λ DNA Ladder (= 50ng/µl), 1 -37 samples

Since the Lambda DNA ladder is standardized at 50ng/ul, it therefore shows that all the samples extracted had DNA above this threshold and because the minimum recommended DNA concentration for conventional PCR is 20ng/ul, the quantity of test samples was therefore above recommended value.

Nanodrop readings for DNA concentration ranged between 93 and 1972 ng/µl, which were enough concentrations for conventional PCR and sequencing methods used in this study. The 260/280 ratios of the extracted DNA ranged between 1.32- 2.01 indicating that the DNA was of high purity and integrity because the values fall within pure DNA ranges recommended for this kind of study using the applied technology (Table 3)

Table 2. Quantification of microbial genomic DNA using nanodrop spectrophotometer

Sample Number	Isolate code	Nucleic acid conc. (ng/µl)	260/280 (purity)
1	$M^{1}SC$	441.4	1.72
2	$A3^{5}$	590.2	1.72
3	A38	582.6	1.64
4	$M^{2}C$	839	1.57
5	$A^{6}2$	678.2	1.61
6	$R^{3}SC$	378.4	1.72
7	A14	952.4	1.63
8	A^{6} 4	585	1.78
9	B2SW	341.5	1.72
10	$M^{1}2C$	382.8	1.71
11	A39	343.1	1.65
12	$A^{6}17$	171.2	1.57
13	D2C	773.3	1.58
14	$J^{6}1$	1386.9	1.32
15	A21	591.3	1.66
16	$R^{3}2C$	369	1.69
17	$A^{6}27$	546.9	1.69
18	R2SRW	93	1.64
19	$B^{4}1W$	290.3	1.7
20	$A^{6}5W$	742.1	1.68
21	A22	602	1.66
22	$B^{3}1SW$	419.9	1.72
23	$A^{5}4$	458.7	1.72
24	$A^{6}8$	6102.3	2.01
25	DW	1595.1	1.55
26	A36	943.3	1.67

Table 3: continued….			
27	$M^{1}2W$	713.2	1.56
28	$B^{6}2W$	347.8	1.72
29	M3W	436.8	1.76
30	$M^{1}1C$	1972.2	1.64
31	$A^{4}9$	967	1.66
32	$A^{6}10$	398.7	1.65
33	$A^{6}13$	279.9	1.67
34	$A^{6}5C$	409.1	1.75
35	$R^{3}1SW$	168.3	1.69
36	$B^{4}1SW$	488.2	1.64
37	$A^{6}37$	386.1	1.7

Key: A - Commercial source, Other first letters – Household sources, SW– small white colony, W – White colony, C – Cream white colour colony, Conc. – Concentration of DNA, subscript – dilution of culture.

4.3.2 Identification of isolates by 16S rDNA sequencing

Amplifications of the V3 region of the bacterial 16S rRNA gene by polymerase chain reaction assay using a group-specific primer (Lac 1 and Lac 2) for the detection of members of the genera *Lactobacillus, Leuconostoc, Pediococcus*, and *Weissella* only amplified three isolates; sample number 24 ($A^{6}8$), 27 ($M^{1}2W$) and 28 ($B^{6}2W$) whose 16S rDNA sequence data is presented in Table 4 below.

The resultant nucleotide sequences (results) were searched for homology with known sequences in the NCBI database using BLAST. The results presented in Table 4 indicated that the search query of the nucleotide sequences of the 5 strains was aligned to rDNA sequences of different species belonging to three groups (genera), namely, *Lactobacillus* (24 ($A^{6}8$) and 27 ($M^{1}2W$)), *Epicoccum* (6 ($R^{3}SC$) and 9 (B2SW)), and *Staphylococcus* 28($B^{6}2W$) with identities of over 97%. At species level, the *Lactobacillus* were mostly *L. plantarum*, *Epicoccum* were *E. nigrum* and for *Staphylococcus*, it was *S. warneri* although it was difficult to discern at strain level using the rDNA sequencing.

Table 4. Genotypes of the 5 isolated microorganisms by ribosomal DNA gene sequence alignments submitted to the NCBI Gene Bank database (BLAST)

Strain No.	Strain Name	Gene bank Acc. No.	Query coverage (%)	Max. ident. (%)
24 ($A^6$8) Lac 1	Uncultured *Lactobacillus* spp.	JF427675.1	98	99.02
24 ($A^6$8) Lac 2	*Lactobacillus plantarum* strain LPP19	KY614058.1	99	99.34
27 ($M^1$2W) Lac1	*Lactobacillus plantarum* strain syn3	KM023152.1	94	98.33
27 ($M^1$2W) Lac2	*Lactobacillus plantarum* DCRUST PKLP4	MH548358.1	95	98.33
28 ($B^6$2W) Lac 1	*Staphylococcus warneri* strain IAE237	MK414942.1	95	98.66
28 ($B^6$2W) Lac 2	*Staphylococcus warneri* strain A28	MK712427.1	97	99.01
6 (R^3SC) ITS-1	Uncultured *Epicoccum* clone SW 2dG08	JF449836.1	96	99.8
9 (B2SW) ITS-4	*Epicoccum nigrum* isolate ECU67	MF435115.1	93	99.16
9 (B2SW) ITS-1	*Epicoccum nigrum* isolate AS-S-19	MK632017.1	96	97.36
24 ($A^6$8) 907-R	*Lactobacillus plantarum strain UIGOA134*	KY817129.1	60	73.60
24 ($A^6$8) 1492-R	No significant similarity found	-	-	-

The partial rRNA gene sequences (11) were aligned and a phylogenetic tree constructed with phylogenetic positions of the strains was compared in a dendrogram with related taxa (Figure 3). Sample number 24 ($A^6$8) and 27 ($M^1$2W) showed close proximity and were close to *L. plantarum* for all the sequences amplified because they grouped on the phylogenetic tree except for primer 1492-R amplicon that resulted into a sequence that did not match any sequence in the DNA database. Sample number 6 and 9 also showed close proximity and were close to *Epicoccum nigrum* and the partial sequences amplified by the same primer grouped together on the phylogenetic tree (Figure 5. On the other hand, sample number 28 was alone in the phylogenetic tree and its partial 16S rRNA gene sequence showed high identity to *Staphylococcus warneri.* In all the cases, it was difficult to discern the isolates at strain level.

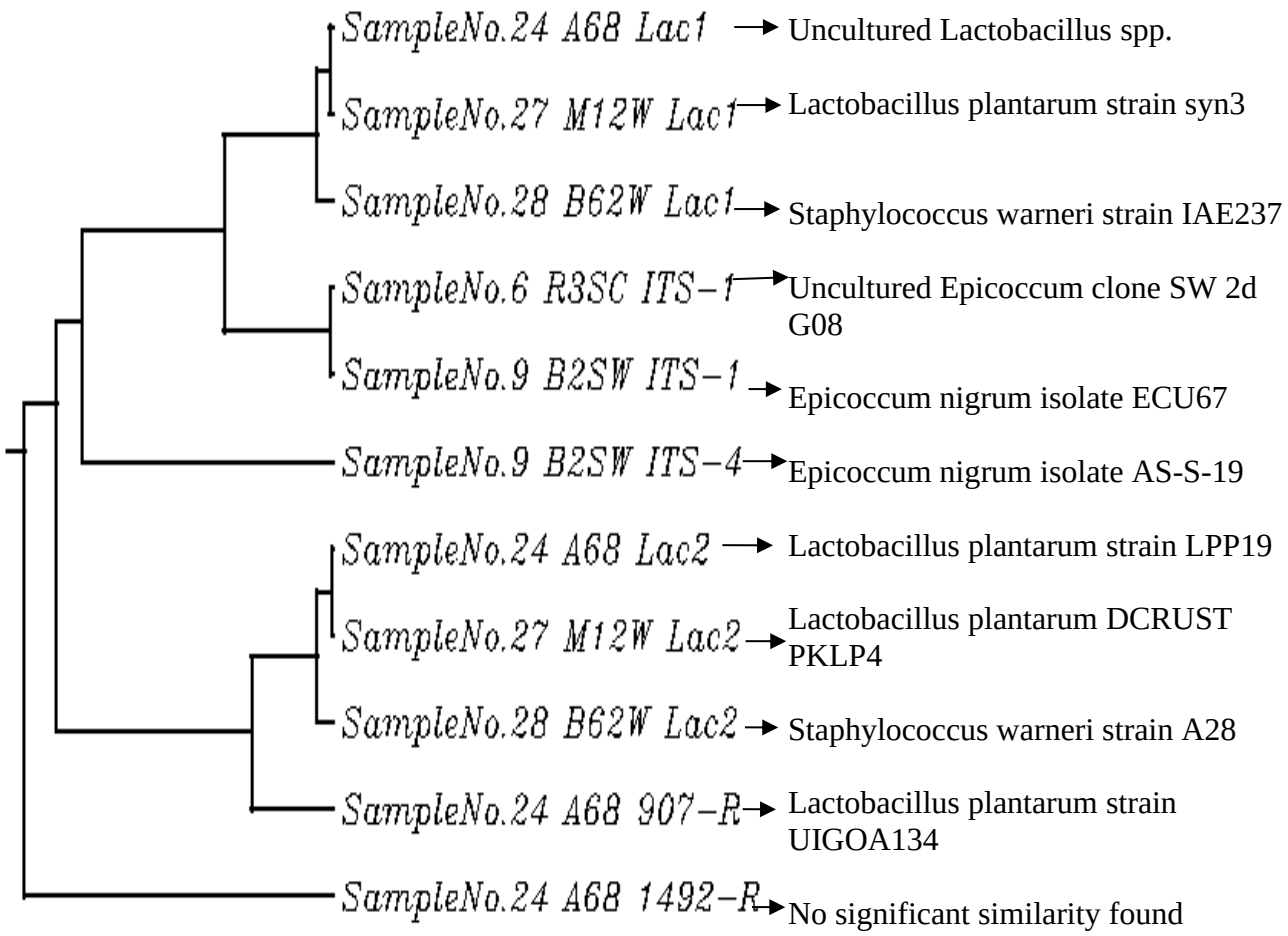
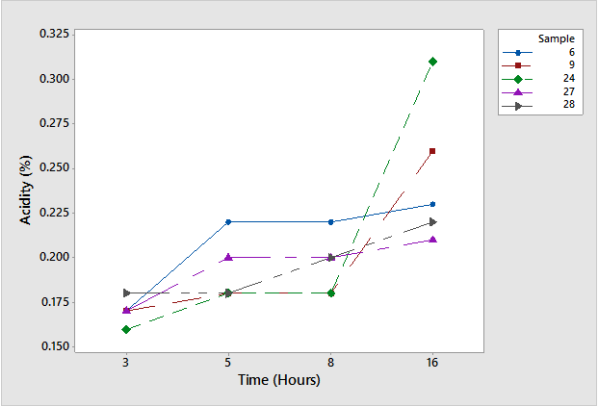


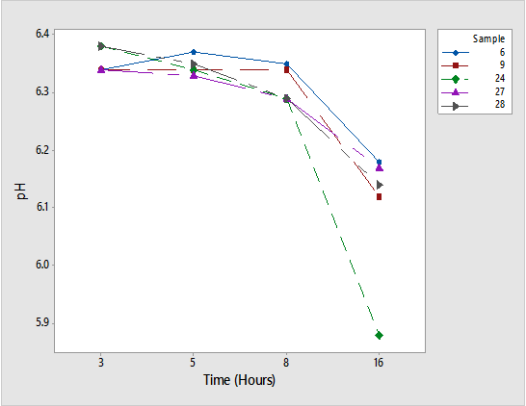
Figure 5: Neighbor-joining tree showing the phylogenetic relationships between isolates of *Lactobacillus plantarum*, *Epicoccum nigrum* and *Staphylococcus warneri* species based on rRNA genes.

4.4 Fermentation capability of the isolates as potential starter cultures for product development

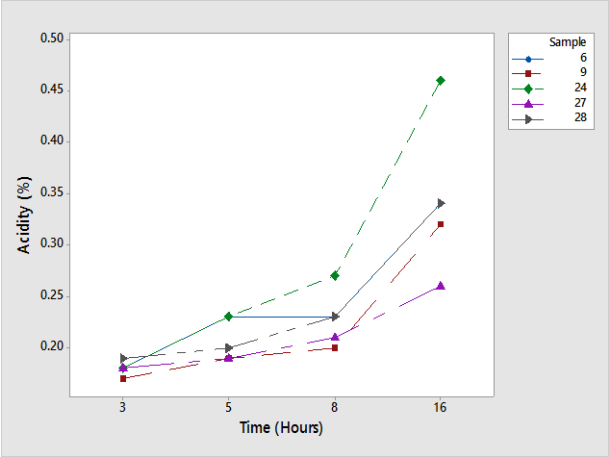
The identified isolates were analyzed for their performance as potential starter cultures. Their acid production and pH change with time were determined at 24ºC, 37ºC and 45ºC (Figure 6). In the time course experiment, the results revealed that the acidifying activity (technological criteria) of *L. plantarum* isolate number 24 was significantly higher than the activity of the other species over a 16 h period and this corresponded to a faster drop in pH over the same period of time. However, the highest acidity attained at this temperature was 0.31% and a pH of 5.85. At 37°C, *L. plantarum*, strain number 24 was the fastest in acidifying activity and from 0-8 h, the rate of acid production was faster than at 24°C and from the 8^{th} h towards the 16^{th} h, acidifying activity was faster and reached 0.47%, which was significantly higher than 0.31% reached at 24°C. At 37°C, the pH also dropped to 4.95 for *L. plantarum* (number 24) and was more acidic than 5.85 attained at 24°C by the same strain. *L. plantarum* (number 27) although identified as the same species as number 24, was slower in acidifying activity than number 27. At 45° C, *L. plantarum* number 24 was still the fastest in acidifying activity reaching 0.4% after 16 h with a corresponding pH of 5.05, signifying that the organism had better acidifying activity at 37°C than at 45°C and 24°C. Strain number 6 identified as *Epicoccum nigrum* was found to be second best in acidifying activity especially at 45°C. From these preliminary studies, 37°C was selected for product development using the *L. plantarum* strains (numbers 24 and 27).



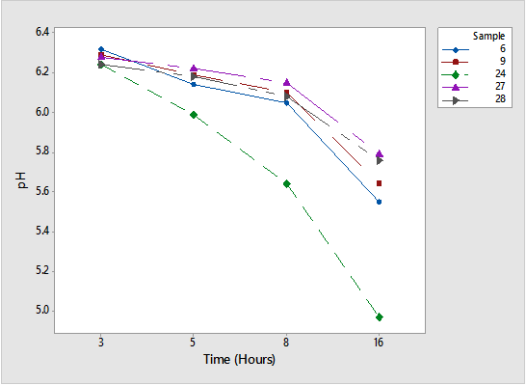
A



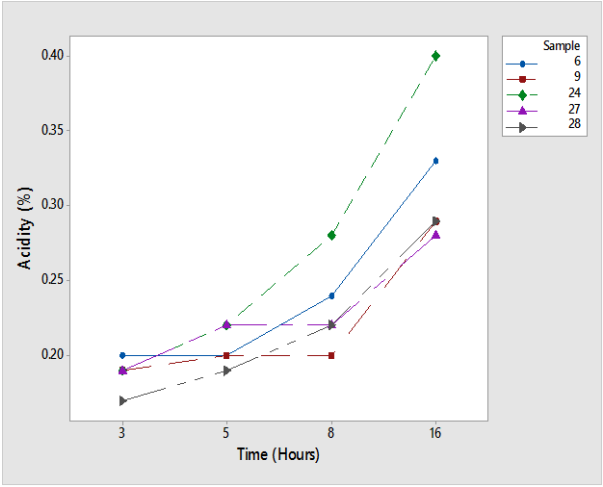
B



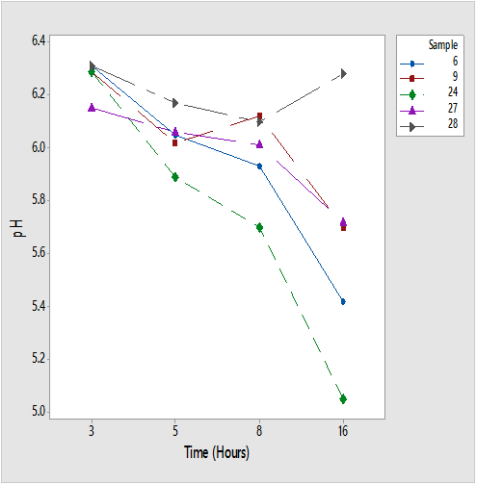
C



D



E

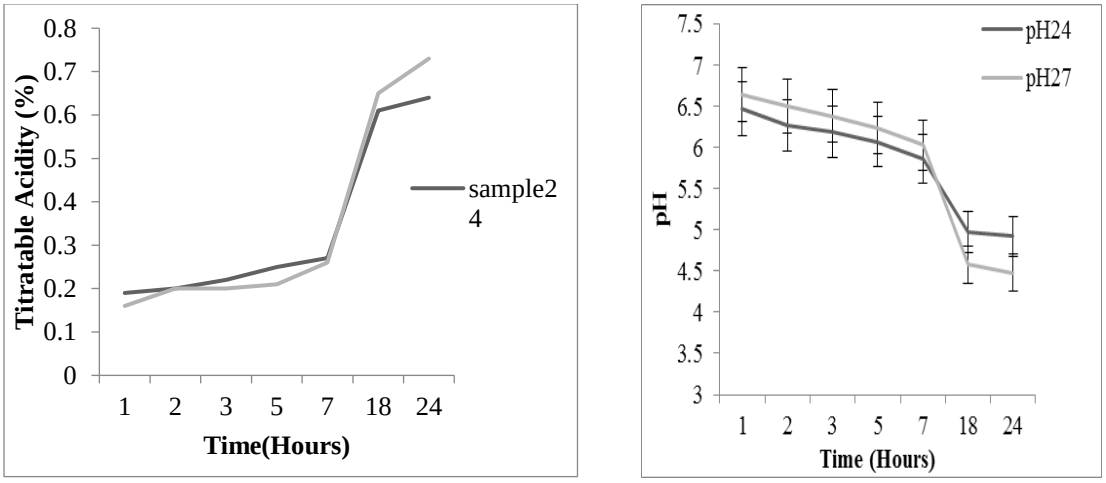


F

Figure 6: Time course graphs showing acidity development (A, C and E)) and pH change (B, D and F) for the different isolates at 24°C (A and B) 37°C (C and D) and 45°C (E and F).

4.5 To determine the fermentation capability of lactic acid bacteria isolated for use as potential starter culture for product development.

The two *L. plantarum* strains were cultivated at 37°C and acidifying activity evaluated at this optimum temperature over a period of 24 h (Figure 7). It was observed that the strains were similar in their acidifying activity and were clotting the milk, characteristic of fermentation. They were both slow for the first 7 h, after which acid production was rapid up to 18 h where production slowed and it reached 0.64% for strain number 24 and 0.73% for strain number 27 after 24 h (Figure 5A). The increases in titratable acidity corresponded to pH drop that slightly changed in the first 7 h but thereafter dropped steadily until it reached 4.48 and 4.92 for strains number 27 and 24, respectively after 24 h of cultivation (Figure 7B).



A B.

Figure 7: Acid production (A) and pH change by the two strains of *L. plantarum* isolated from a*mabere amaruranu* at 37ºC. Sample No. 24 and sample No. 27 = the two isolates used. pH 24 = Isolate No. 24 pH change curve; pH 27 = Isolate No. 27 pH change curve.

CHAPTER FIVE

DISCUSSION

5.1 Microbiological profiling and enumeration of the initial microbial load

Both sources of *amabere amaruranu* had high total microbial loads far above the KEBS maximum standard of 2×10^4 cfu/ml TVC/ml for Kenyan cultured fermented milk products (KEBS DKS05-941: 2013, 2018). TVC for household's source was slightly over 10^4cfu/ml while commercial milk processors source was over 10^{10} cfu/ml as shown in Table 1. From the results, the high TVC for both the households and commercial processors could be associated with production and handling practices. It is possible the milk used in the production was not adequately boiled. The Kenyan standard specification for cultured fermented milks,(KEBS DKS 941: 2013, 2018), gives other microbiological as follows; total coliforms counts as maximum of 10 cfu/ml or g, total yeasts and moulds counts of 100 cfu/ml or g. Table 1 gives total coliforms counts as $\log_{10}$ 7.6 for commercial producers and $\log_{10}$ 6.57 for households, and total yeasts and molds counts as $\log_{10}$ 8.09 for commercial producers and $\log_{10}$ 6,2, these counts are far above the standard microbiological requirements. Hence both sources of amabere amaruranu do not comply with KEBS requirements which compromises the safety and quality of the product.

During the process of fermentation, the lactic acid bacteria continue multiplying in *amabere amaruranu* contributing to the elevated high numbers. However, milk processors had higher counts compared to the household. This can be attributed to the fact that the households after milking they immediately or take a shorter time before putting in the fermentation guards or containers. On the other hand, those commercial milk processors take longer time before the milk reach to them allowing multiplication. Also, the milk that has not been purchased for the day is what is used for fermentation when its start going bad. This can also be a cause of the higher counts. Similarly, it is spontaneous fermentation and therefore there is no control over microbial growth during the fermentation process.

During spontaneous fermentation, the organisms involved are mostly *Lactobacillus spp.* as reported in studies done on spontaneously fermented milk in the Kenya and Sudan by Tambekar & Bhutada (2010) and Lore *et al.* (2005). These authors reported that the LABs groups of bacteria, especially *Lactobacillus spp.* are commonly encountered in traditional fermented milk. The mean difference between TVC and coliforms can further be associated by the high content of LABs in *amabere amaruranu.* The LABs metabolize sugar in the milk via a number of fermentation

pathways to produce carbon dioxide, lactic, acetic and propionic acids. These acids in turn lowers the pH of the increased acidity in provides *amabere amaruranu* unfavourable environment for the growth of the pathogens. Acids perform antimicrobial activity through interference with integrity of the cell membrane, reducing intracellular pH and inhibiting active transport and other metabolic functions (Dardir, 2012; Rattanachaikunsopon & Phumkhachorn, 2010). However, the higher TVC microbial counts indicate the milk might have been highly contaminated and poor hygiene and handling practices total coliform counts were higher for commercial milk processors ($\log_{10}$ 7.60cfu/ml) compared to the households ($\log_{10}$ 6.57cfu/ml). This generally indicates poor hygiene among the actors when handling the milk. Commercial milk processor had higher contamination which may have resulted from contamination during transportation. The transportation containers may have biofilms which continuously contaminate the milk (Wafula *et al.,* 2016). Also, milk used by the processor sometimes is added contaminated water that further contaminates the milk. Similarly, coliforms in the *amabere amaruranu* could be associated with contaminated containers surfaces, dust and soil that may contaminate the milk during milking process. The coliforms exist on plant materials and in the soil, and can be spread into the atmosphere through dust. Since coliforms are both of faecal and non-faecal origin, they have the ability to multiply outside the surface of containers, hence their presence *amabere amaruranu.* This is therefore the original source of contamination by coliforms cannot be definitively ascertained but it is likely they arose out of improper handling and transferring of *amabere amaruranu* from one container to the next during bulking.

Moreover, contamination may also from those individuals who don't observe proper hygiene and hence *amabere amaruranu* being exposed to contamination in many ways. Some of the microorganism such as *Escherichia coli* are resistant to pH and hence survive in the milk despite the acid development. Coliforms outsmart the natural antimicrobial proteins in *amabere amaruranu* and the secondary metabolite organic acids produced after substrate breakdown of LABs synthesis because they can adapt to several survival strategies in the milk. The strategies employed by these Gram-negative rods include acid tolerances, temperature evasions and production of complex and cooperative colonies. Other strategies genetically controlled (Adams & Moss, 1997) *amabere amaruranu* samples were highly contaminated with yeasts and moulds. These might be possible due to poor processing conditions and / or uncontrolled fermentation which lead to contamination with yeasts and moulds and this obvious by alcoholic production in

addition to lactic acid. Yeasts and moulds are frequently isolated and are present in milk product. *Epicoccum nigrum* which is known as black yeast found on paintings and wall paper cotton and textiles, in dust, and in air and is tolerant of changes in water availability (Singh, 1994), was found to be present in the samples where isolates were taken. This clearly indicates that the environment plays a key role in the quality of the product. Yeast and moulds survive well under low pH hence able to thrive in the spontaneously fermented milk.

5.2 Morphological and Physiological identification of lactic acid bacteria

The isolates were cultured at different temperatures to help identify whether they were psychrophilic, mesophilic or thermophilic groups. Psychrophilic microbes grow at temperature less than 15ºC and optimum at 20ºC. Mesophilic group can grow at warmer temperature, ranging from 15ºC to 45º C, while thermophilic group can grow at high temperature, which is between 45ºC and 80º C. More than 90% of the isolates were able to grow at 15ºC, 34.1% were able to grow at 45ºC while 65.9% were able to grow at 6.5% salt concentration (Table 2).

This examination gave an indication of the osmotolerance level of the LAB strains. According to Ibourahema *et al*. (2008), bacterial cells cultured with a high salt concentration could show a loss of turgor pressure, which would then affect their physiology, enzyme activity, water activity and metabolism. According to Adnan and Tan (2007), high osmotolerance would be a requirement of LAB strains to be used as commercial strains, because when lactic acid is produced by the strain, alkali would be pumped into the broth to prevent an excessive reduction in pH, and the free acid would be converted to its salt form, increasing the osmotic pressure on the bacteria. In this study computations from table 2, 81.8% were able to grow at 4.0% salt concentration. Most of the isolates (65.90%) because they were able to grow at high salt concentration hence salt tolerant. Hence, most of the strains were fit for use as starter culture for production purpose.

5.3 Molecular characterization of lactic acid bacteria

The quantity of DNA was measured by the nucleic acid values while the quality of the DNA was measured by 260/280 values which the acceptable values ranging from 1.50 – 2.2 (Table 2) where pure DNA should be 1.8 while pure RNA should be 2.0. Therefore, the quantity and quality of DNA extracted from the isolates was within the acceptable limits for PCR amplification and sequencing. On PCR for sequencing, only five (5) isolates were able to give amplicons that were sequenced even with extra primers that were employed. The samples were stored for a long

time (one month) after DNA extraction in extraction buffer before amplification and sequencing. This long storage could have led to hydrolysis of the DNA which could have lowered its quality leading to poor sequencing results. Additionally, according to Munshi (2012), there are some common automated DNA sequencing problems such as Failure of the DNA sequencing reaction resulting from many technical problems, mixed signalling resulting in multiple peaks because of multiple amplification banding or background DNA, poor-quality data and short read lengths and excessive dye blobs which is an inherent problem associated with Sanger sequencing, the technology applied in this study. Formation of primer dimers in sequence reaction and slippage of DNA-polymerase on the template are also commonly reported sequencing problems. Failure to amplify and sequence most of the DNA extracted from most of the isolates could be attributed to any of these factors.

The partial DNA sequence for isolate No. 24 and 27 in appendix 2 identified them as *Lactobacillus plantarum.* Isolate No. 6 and 9 were also identified as *Epicoccum* spp. while isolate number 28 had close proximity to *Staphylococcus warneri*, which is a pathogen. With the rDNA sequencing, it was difficult to discern the isolates at strain level, showing the limited discriminatory power of the method for identification to strain level, hence species-specific PCR and PCR-RFLP analysis could serve as an alternative method to barcode the isolates at the species or strain level. The isolates in this study were first cultivated on MRS media that is routinely used for the isolation and counting of LAB from most fermented food products. In Table 2, isolate No. 6 and 9 had already been designated as rods and short rods, respectively, based on morphological and physiological characteristics. However, MRS medium is known to exhibit poor selectivity for *Lactobacilli* (Ouadghiri *et al*., 2005), hence the detection of fungus on a *Lactobacillus*-specific MRS media.

Lactobacillus plantarum was the main LAB isolated in this study. Among the *Lactobacillus* species, *L. plantarum* is a versatile species that has useful properties and is mostly found in an array of fermented foods (Guidone *et al*., 2014). For instance, Mathara *et al*. (2004) found *L. plantarum* to be the major *Lactobacillus* species in *kule naoto*, where it was thought to be responsible for the product characteristics. Moreover, the species has been reported in *mursik* (Digo, 2015) and is also the most prevalent *Lactobacillus* species in *amabere amaruranu* and was reported to comprise of 20% of the total isolates (Nyambane *et al*., 2014), which implies that the species plays a vital role in the fermentation of *amabere amaruranu*. *L. plantarum* is widely

applied in fermentation and processing of raw foods industrially and is “generally recognized as safe” (GRAS) and has qualified presumption of safety (QPS) (Ray and Joshi, 2014; Park *et al*., 2016). With the continued growth of the market for fermented ingredients and foods coupled with increased consumer demand for food healthy products, the isolate will definitely help is the design of healthy foods, because the strain has been described to have known probiotic properties. For probiotic properties, the aantibacterial activity of *L. plantarum* strains toward different pathogens and food spoilage bacteria have been widely analysed and depends on the *L. plantarum* strain and pathogen strain as well as culture conditions. There are various mechanisms and molecules involved in the relationship between pathogens and probiotics such hydrogen peroxide, organic acids, ethanol and production of bacteriocin (Margolles *et al*., 2009). Only a few studies have dealt with potential antiviral activity: one related with herpes simplex (HSF) strain F (Todorov *et al*., 2008) and other against influenza infection in mice (Takeda *et al*., 2011). Antifungal activity against moulds and yeasts are widely reported, most of them in dough and bakery products as well as in some cheeses (Djossou *et al*., 2011). Moreover, the strain has an outstanding effect on the flavor and texture in resulting fermented foods (Adesulu-Dahunsi, 2017); hence ease of acceptability of *Lactobacillus plantarum*-fermented food products.

Epicoccum nigrum which is known as a black yeast found on paintings and wall paper cotton and textiles, in dust, and in air and is tolerant of changes in water availability (Singh, 1994), was found to be present in the samples where isolates were picked from. This clearly indicates that the environment where milk is produced significantly contributes to the quality of the product. Yeast and moulds survive well under low pH hence able to thrive in the spontaneously fermented milk. The interest in *Epicoccum* sp. could be in its vast secondary metabolites which include polyketide, polyketides, diketopiperazine and hybrids, that it produces. Many of these metabolites exhibit bioactivities, such as antioxidant, antimicrobial, anticancer, and antiviral properties. This ability makes *Epicoccum sp* important producers of biological compounds of potential medical applications. *Epicoccum sp* also produce epicocconone a compound used as a cell stain during protein detection in gel electrophoresis (Bell and Karuso, 2003). *Epicoccum nigrum* has been exploited to produce the telomerase inhibitor D8646-2–6 (Kanai *et al*., 2007). And taxol, which is an anticancer (Somjaipeng *et al*., 2016). All these show that although *E. nigrum* is plant pathogen and a contaminant in fermented milk, its benefits or threats in fermented milk are yet to be

determined. However, it could confer health benefits and has potential application as a probiotic in milk and possibly other food products.

Isolate No. 28 was identified as *Staphyococcus warneri*, which is a coagulase-negative *Staphylococci* (CNS). CNS species are naturally occurring harmless skin commensals, or opportunistic pathogens of low virulence (Huebner and Goldmann,1999). CNS species may be found in fermented foods (Coton *et al*., 2010). For instance, they are commonly found in fermented meats and sausages, but their presence is usually considered as safe. *S. warneri* species have also been found in French cheeses, dry fermented sausages, processing environments and clinical samples. Three strains of *Staphylococcus warneri* isolated from meat samples were found to produce warnerin, a bacteriocin; which inhibits the growth of bacterial growth, hence the strains of *S. warneri* have potential as probiotics (Prema *et al*., 2006). Its presence in *amabere amaruranu* could be associated with contamination from the cows or human handlers; however, its role in the milk as a pathogen or as a probiotic need to be investigated.

5.4 Acidifying Activity of the identified Isolates

For this study, technological criterion was used to select all the isolates that were satisfactorily identified by rDNA sequencing. Acid production properties of LABs are exploited as technological characteristics in the dairy industry (Berresford *et al.*, 2001). For this aim, pH change and lactic acid production were monitored for the five isolates and it was found that acid production (titratable acidity) increased with time while pH reduced with time as is characteristic of LAB. LAB ferments the milk carbohydrate (lactose) to produce lactic acid that lead to decrease in pH and increases the value of titratable acidity. The obtained results revealed that isolate No. 9 performed as a better lactic acid producer as well as isolate No. 6, which are fungal strains (*Epicoccum* spp.) and isolate no. 28 (*Staphyococcus warneri*). Despite their acidifying activity, they were disqualified and isolates No. 24 and 27 (both *Lactobacillus plantarum* strains) were further evaluated for their acidifying activity. Results obtained showed that the two *Lactobacillus plantarum* strains had a similar trend in acidification but neither could be characterized as fast, as they didn't reach acidity of 0.4% in 3h at the optimum growth temperature (El Soda *et al*., 2003). These results were in agreement with those reported by Durlu-Ozkaya *et al*. (2001), where it was found that *Lactobacillus* strains differ in their ability to reduce milk pH initially and there are some strains that don't change the pH of milk after 6 h of inoculation. *Lactobacillus plantarum* are known to produce Lactic acid without inhibition and are facultative and heterofermentative

microbes. Fermentation of sugars result in lowering of pH which is important in milk coagulation. Besides, increasing acidity catalyses reactions and changes that leads to desired texture and flavour in fermented milk and also whey expulsion. The two isolates were found to grow at high temperatures of 45^{o}C. According to Ibourehama *et al.* (2008), the capacity of bacteria to grow at high fermentation temperatures is advantageous in indicating a likely increased rate of lactic acid production and growth. Furthermore, the ability of these isolates to grow at high fermentation temperatures could decrease contamination by other microorganisms especially the pathogens. From the study by Yelnetty *et al*. (2014), the proteolytic bacteria, *Lactobacillus plantarum* have the ability to ferment milk and could be used as starter culture to produce fermented milk both at household and commercial level.

Coagulation of the milk by the two *Lactobacillus plantarum* isolates (as starter cultures) was examined together with acid development. It was observed (Figure 7A) that coagulation of the milk by the two starter cultures started before 5 h and the milk became a firm coagulant in 18 hours to 24 hours. At the early stages of fermentation, the pH value of each milk varied in the range of 6.47 to 6.64, which is typical of raw milk and decreased to the range of 4.93–4.48 after 24 h of fermentation. Findings by Seelee *et al*. (2009) showed that pH value decreased during milk fermentation and the longer the fermentation time, the lower the pH value will be. Pathogenic microorganisms which can cause disease, food poisoning and spoilage are inhibited by the low of the pH to below 4.0 resulting from acid production (Ananou *et al.*, 2007). Therefore, the use of the isolated *Lactobacillus plantarum* strains as starter cultures will require fermentation of more than 24 h to further reduce the p^{H}. This will prolong the shelf life of the fermented milk. However, *Lactobacillus plantarum* is a microorganism used mainly as a probiotic (Ukeyima *et al*., 2010). Probiotics are life microorganisms which when consumed have health benefits to the consumer. Numerous researches have focused on role of fermented foods as anticarcinogenic with positive results. *Lactobacillus plantarum* hence have potential in reducing or even eliminating alimentary canal procarcinogens and carcinogens. (Mital & Garg, 1995; Reddy *et al.*, 1983), production of bacteriocins, antibacterial and antifungal properties, antioxidant, antimutagenic and health promoting properties. Therefore, the isolated strains could be used as a starter culture to produce their own products, or they can be incorporated in other fermented products to confer the probiotic properties.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i. It is possible to characterise microbial diversity of *amebere amaruranu* using both cultural and molecular techniques. *amabere amaruranu* from both sources contain diverse microbial populations in terms of type (moulds, lactic acid bacteria, yeasts and coliforms) and numbers. This information is very key when assessing safety and quality of *amabere amaruranu*. High numbers of coliforms may present a health hazard hence safety of the product need to be addressed by standardizing the process of production of the spontaneously fermented milk using a starter culture.

ii. Molecular characterization results confirmed that *Lactobacillus plantarum* was the most predominant Lactic acid bacteria in *amabere amaruranu* that is identified as a potential starter culture to upgrade the production of product.

iii. *Lactobacillus planturum* strains coagulated the milk and reduced the pH of milk showing fermentation capabilities hence could be used as starter culture for product development.

6.2 Recommendations

i. There is need for more research that can use strain-specific PCR and bacterial barcoding technology which could serve as an alternative method to discriminate the isolates at the strain level.

ii. Application of *insitu* sequencing technologies such as pyrosequencing which involve sequencing directly from samples without DNA isolation could be more successful in identification of potential starter cultures.

iii. Further research on the identified *Lactobacillus plantarum*'s fermentation capability aspects such viability and stability at different fermentation conditions and other processability conditions should be done.

REFERENCES

Abdelgadir, W.S., Hamad S.H., Møller P.L. and Jakobsen M. (2001). Characterization of the dominant microbiota of Sudanese fermented milk Rob. *International Dairy Journal*, *11*, 63–70.

Abdullah, S. A. and Osman, M. M. (2010). Isolation and Identification of Lactic Acid Bacteria from Raw Cow Milk, Cheese and Rob in Sudan. *Pakistan Journal of Nutrition*, *9*, 1203-1206.

Adams, M.R. and Moss M.O. (1997). *Microbiology of Food*. Translated by Manuel Ramis Vegés; by Editorial Acribia, Royo, 23 50006 Zaragoza, pp 464. ISBN 84 200 0830 3.

Adesulu-Dahunsi, A. T., Sanni, A. I., Jeyaram, K. and Banwo, K. (2017). Genetic diversity of *Lactobacillus plantarum* strains from some indigenous fermented foods in Nigeria, *LWT-Food Science and Technology*, *82*, 199–206.

Adnan, A. F. M. and Tan, I. K. (2007). Isolation of lactic acid bacteria from Malaysian foods and assessment of the isolates for industrial potential. *Journal of Bioresource Technology*, *98*(7), 1380-1385.

Akabanda, F., Owusu-Kwarteng, J., Tano-Debra, K. and Glover, R. L.K. (2013). Taxonomic and molecular characterization of lactic acid bacteria and yeasts in nunu, a Ghanaian fermented milk product. *Food Microbiology***,** *34*, 277–283

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research*, *25*(17), 3389-3402.

Amor, K. B., Vaughan, E. E. and de Vos, W. (2007). Advanced molecular tools for the identification of lactic acid bacteria. *Journal of Nutrition*, *137*, 741-747.

Ananou, S., Maqueda, M., Martínez-Bueno, M. and Valdivia, E. (2007). Biopreservation, an ecological approach to improve the safety and shelf-life of foods. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology, 1*, 475-487.

Ashton, Q. A. (2013). *Lactobacillus - Advances in Research and Applications.* Atlanta, Georgia: Scholarly Editions pp 456-487.

Atanda, O. O. and Ikenebom, M. J. (1989). Effect of heat treatments on the microbial load of nunu. *Letters in Applied Microbiology, 9*, 233-235.

Barakat, O. S., Ibrahim, G. A., Tawfik, N. F., El-kholy, W. I. and Gad el-rab, D. A. (2011). Identification and probiotic characteristics of Lactobacillus Strains isolated from traditional domiati cheese. *International Journal of Microbiological Researc*h, *3***,** 59-66.

Bell, P. J. and Karuso, P. (2003). Epicocconone, A Novel Fluorescent Compound from the Fungus E*picoccum nigrum*. *Journal of the American Chemical Society*, *125*(31), 9304-9

Beukes, E. M., Bester B. H and Mostert J. F. (2001). The microbiology of South African traditional fermented milks. *International. Journal of Food Microbiology, 63*, 189–197.

Botina, S. G., Tsygankov Y.D. and Sukhodolets, V. V. (2006). Identification of industrial strains of lactic acid bacteria by methods of molecular genetic typing. *Russian Journal of Genetics, 42*, 367-1379.

Boyiri, B. B. (2014). Probiotic Potential of Bacterial Isolates From 'amabere amaruranu' Cultured Milk, CRC press, pp 101-124.

Britton, R. A. and Versalovic, J. (2008). Probiotics and gastrointestinal infections. *Interdisciplinary Perspectives on Infectious Diseases, 2018,* 290- 301.

Campbell-Platt, G. (1994). Fermented foods—a world perspective. *Food Research International*, 27(3), 253-257.

Chelule, P. K., Mokoena, M. P. and Gqaleni, N. (2010). Advantages of traditional lactic acid bacteria fermentation of food in Africa. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*, 2, 1160-1167.

Chichlowsk, M., Croom, J., McBride, B. W., Havenstein, G. B. and Koci, M. D. (2007). Metabolites and Physiological impacts of probiotics or direct-fed microbial on poultry: A brief review on current knowledge. *International Journal of Poultry Science, 4*: 889 - 896.

Chilton, S., Burton, J. and Reid, G. (2015). Inclusion of fermented foods in food guides around the world. *Nutrients*, *7*(1), 390-404.

Chung, S. K., Mee, S. L., S. I. and Sang, C. P. (2010). Discovery of Novel Sources of Vitamin B12 in Traditional Korean Foods from Nutritional Surveys of Centenarians. *Current Gerontology and Geriatric Research, 3*, 374-397.

Clarridge, J. E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews*, *17*(4), 840-862.

Cocolin, L., Aggio, D., Manzano, M., Cantoni, C. and Comi, G. (2002*). An application of PCR-DGGE analysis to profile yeast populations in raw milk. International Dairy Journal*, *12*, 407-411.

Cocolin, L., Rajkovic, A., Rantsiou, K. and Uyttendaele, M. (2011). The challenge of merging food safety diagnostic needs with quantitative PCR platforms. *Trends in Food Science and Technology*, *22*, S30-S38

Coton, E, Desmonts, M. H. and Leroy, S. (2010). Biodiversity of coagulase-negative staphylococci in French cheeses, dry fermented sausages, processing environments and clinical samples. *International. Journal of Food Microbiology*; *137*, 221–229.

Coton, E., Desmonts, M. H., Leroy, S., Coton, M., Jamet, E., Christieans, S. and Talon, R. (2010). Biodiversity of coagulase-negative staphylococci in French cheeses, dry fermented sausages, processing environments and clinical samples. *International Journal of Food Microbiology*, *137*(2), 221-229.

Dardir, H. A. (2012). *In vitro* evaluation of probiotic activities of lactic acid bacteria strains isolated from novel probiotic dairy products. *Global Veterinaria*, *8*(2), 190-196.

Dellaglio, F., Felis G. E. and Castioni, A. (2005). *Lactobacillus delbrueckii* subsp. indicus subsp. nov., Isolated from Indian Dairy Products. *Journal of Evolutionary Microbiology*. *55,* 401–404.

Djossou, O., Perraud-Gaime, I., Mirleau, F. L., Rodriguez-Serrano, G., Karou, G., Niamke, S. and Roussos, S. (2011). Robusta coffee beans post-harvest microflora: *Lactobacillus plantarum* sp. as potential antagonist of *Aspergillus carbonarius*. Journal of *Anaerobe*, *17*(6), 267-272.

Drancourt, M., Bollet, C., Carlioz, A., Martelin, R., Gayral, J. P. and Raoult, D. (2000). 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *Journal of Clinical Microbiology*, *38*(10), 3623-3630.

Durlu-Ozkaya, F., Xanthopoulos, V., Tunail, N. and Litopoulou-Tzanetaki, E. (2001). Technologically important properties of lactic acid bacteria isolate from Beyaz cheese made from raw ewes' milk. *Journal of Applied Microbiology, 91(5*), 861-870.

Elaine, M. and Danilo, M. (2012). *Diversification booklet number 21: Traditional fermented food and beverage for improved livelihoods*. Rome: Food and Agriculture Organization (FAO) of the United Nations.

El-Baradei, G., Delacroix-Buchet A. and J. C. Ogier. (2008). Bacterial diversity of traditional zabady fermented milk. International *Journal of Food Microbiology, 121,* 295–301.

Endo, A. and Okada, S. (2005). Monitoring the lactic acid bacterial diversity during shochu fermentation by PCR-denaturing gradient gel electrophoresis. *Journal of Bioscience and Bioengineering, 99,* 216-221.

El Soda, M., Ahmed, N., Omran, N., Osman, G. and Morsi, A. (2003). Isolation, identification and selection of lactic acid bacteria cultures for cheesemaking. *Emirates Journal of Food and Agriculture, 15*,51-71.

Facklam, R., Hollis, D. and Collins, M. D. (1989). Identification of Gram-Positive Coccal and *Cocobacillary vancomicin*-Resistant Bacteria. *Journal of Clinical Microbiology*; *27,* 724–730.

FAO. (2010). *The state of food insecurity in the world: Addressing food insecurity in protracted crises*. FAO.

Fleet, G. H. (2006). The commercial and community significance of yeasts in food and beverage production. In: The yeast hand book, Querol, A. and G.H., Fleet, (Eds). Springer-Verlag, Berlin Heidelberg, pp 1-12.

Fleet, G. H. (2007). Yeasts in foods and beverages: impact on product quality and safety. *Current Opinion of Biotechnology, 18,* 170–175.

Forouhandeh, H., Vahed, S. Z., Hejazi, M. S. and Akbari, M. D. (2010). Isolation and Phenotypic Characterization of Lactobacillus Species from Various Dairy Products. *Current Research in Bacteriology, 3,* 84-88.

Gadaga, T. H. Mutukumira A. N. Narvhus J. A. and Feresu S. B. (1999). A review of traditional fermented foods beverages of Zimbabwe. *International Journal of Food Microbiology, 53,* 1-11.

Gadaga, T. H., Mutukumira A. N. and Narvus, J. A. (2001). The growth and interaction of yeasts and lactic acid bacteria isolated from Zimbabwean naturally fermented milk in UHT milk. *International Journal of Food Microbiology, 68,* 21-32.

Garvie, E.I. (1984). *Taxonomy and identification of dairy bacteria*. In Davies, F.L. and Law, B.A. (Eds.). Advances in the microbiology and biochemistry of cheese and fermented milk. Elsevier Applied Science Publishers, London. pp 35-66.

Gee, J. E., De, B. K., Levett, P. N., Whitney, A. M., Novak, R. T. and Popovic, T. (2004). Use of 16S rRNA gene sequencing for rapid confirmatory identification of Brucella isolates. *Journal of Clinical Microbiology*, 42, 3649–3654.

Glazunova, O. O., Raoult, D. and Roux, V. (2009). Partial sequence comparison of the rpoB, sodA, groEL and gyrB genes within the genus Streptococcus. *International journal of Food Microbiology, pp 34-56.*

Gonfa, A., Fite A., Urga, K. and Gashe, B. A. (1999). The microbiological aspects of ergo (ititu) fermentation. *SINET Ethiopian Journal of Science 3,* 22-23.

Goodfellow, M. and Minnikin, D. E. (1985). *Chemical methods in bacterial systematics* (No. 20). London Academic Press, pp 1-16.

Gremion, F., Chatzinotas A., Kaufmann K., VonSigler W. and Harms H. (2004). Impacts of heavy metal contamination and phytoremediation on a microbial community during a twelve-month microcosm experiment. *FEMS Microbiology Ecology, 48,* 273–283.

Guarner, F., Perdigon, G., Corthier, G., Salminen, S., Koletzko, B. and Morelli, L. (2005). Should yoghurt cultures be considered probiotic? *British Journal of Nutrition, 93,* 783-786.

Guetouache, M. and Guessas, B. (2015). Characterization and identification of lactic acid bacteria isolated from traditional cheese (Klila) prepared from cow's milk. *African Journal of Microbiology Research*, *9*(2), 71-77.

Guidone, A., Zotta, T., Ross, R. P., Stanton, C., Rea, M. C., Parente, E. and Ricciardi, A. (2014). Functional properties of *Lactobacillus plantarum* strains: A multivariate screening study. *LWT-Food Science and Technology*, *56(1*), 69-76.

Hamama A. (1992). *Moroccan traditional fermented dairy products.* In: Ruskin, F.R. (Ed.) Applications of biotechnology to traditional fermented foods. National Academy press, Washington DC, pp 75-79.

Harrigan, W. F. and McCance, M. E. (1976). *Laboratory methods in food and dairy microbiology*. Academic Press Inc. (London) Ltd, pp 123-145.

Holzapfel, W.H. (2002). Appropriate starter culture technologies for small-scale fermentation in developing countries. *International Journal of Food Microbiology, 75 (3),* 197 – 212

Hoque, M. Z., Akter, F., Hossain, K. M., Rahman, M. S., Billah, M. M. and Islam, K. M. (2010). Isolation, Identification and Analysis of Probiotic Properties of Lactobacillus Spp. from Selective Regional Yoghurts. Bangladesh. *World Journal of Dairy and Food Sciences*, *4,* 39-46.

Hosono, A. Wardojo, R. and Otani, H. (1989). Microbial flora in Dadih, a traditional fermented milk in Indonesia, *LWT-Food Science and Technology, 22,* 20-24.

Huebner, J. and Goldmann, D. A. (1999). Coagulase-negative staphylococci: A role as pathogens. *Annual Review of Medicine, 50,* 223–236.

Hugenholtz, P. (2002). Exploring prokaryotic diversity in the genomic era. *Genome biology*, *3*(2),7-8.

Hussain, S., Devers-Lamrani, M., El Azhari, N. and Martin-Laurent, F. (2011). Isolation and characterization of an isoproturon mineralizing Sphingomonas sp. strain SH from a French agricultural soil. *Biodegradation*, *22*(3), 637-650.

Hutkins, R.W.(Eds.) (2006). *Microbiology and Technology of Fermented Foods*. By Hutkins,R.W. (Eds.) Blackwell Publishing, Iowa; pp 18-24.

Ibourahema, C., Dauphin, R. D., Jacqueline, D. and Thonart, P. (2008). Characterization of lactic acid bacteria isolated from poultry farms in Senegal. *African Journal of Biotechnology*, *7*(12), 10-11.

Irlinger, F. (2008). Safety assessment of dairy microorganisms: coagulase-negative *staphylococci*. *International Journal of Food Microbiology, 126,* 302–310.

Isono, Y., Shingu I. and Shimizu S. (1994). Identification and Characteristics of lactic acid bacteria isolated from Masaï fermented milk in Northern Tanzania. *Bioscience Biotechnology Biochemistry, 58,* 660-664.

Jay, J. M. (1992). *Modern food microbiology*. Chapman and Hall, New York. pp. 371-409

Jespersen, L. (2003). Occurrence and taxonomic characteristics of strains of *Saccharomyces cerevisiae* predominant in African indigenous fermented foods and beverages. *FEMS Yeast Research*, *3*(2), 191-200.

Kanai, A., Takeda, Y., Kuramochi K., Nakazaki, A. and Kobayashi, S. (2007). Synthetic study on telomerase inhibitor, D8646-2-6: synthesis of the key intermediate using Sn (OTf)2 or Sc (OTf)3 mediated aldol-type reaction and Stille coupling, *Chemical Pharmaceutical Bulletin*, *55,* 495–499.

KEBS, Kenya Bureau of Standards. (2018). Standard microbiological limits of cultured fermented milks KEBS DKS, 05 –941, Government Press Nairobi, Kenya.1-7.

Kisii County Government. (2019, October 25). *Who we are? Kisii County Position*. Retrieved from Kisii County Government: https://www.kisii.go.ke/.

Kotala, J. and Onyango, E. (2015). *Lactobacillus rhamnosus* Cultured Milk Isolate May Reduce Adipogenesis. *The FASEB Journal*, *29*(1_supplement), 1010

Kunyanga, C. N., Mbugua, S. K., Kangethe, E. K. and Imungi, J. K. (2009). Microbiological and acidity changes during the traditional production of kirario: An indigenous Kenyan fermented porridge produced from green maize and millet. *African Journal of Food, Agriculture, Nutrition and Development*, *9*(6), 127-128.

Kurmann, J.A. (1984). The production of fermented milk in the world: aspects of the production of fermented milks. *International Dairy Federation Bulletin*; *179, 16-26*.

Kurtzman, C. P. and Robnett, C.J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie Leeuwenhoek. *Journal of Genetics, 73,* 331–371.

Latorre-García, L., Castillo-Agudo, L. and Polaina, J. (2007). Taxonomical classification of yeasts isolated from kefir based on the sequence of their ribosomal RNA genes. *World Journal of Microbiology and Biotechnology*, *23,* 785-79.

Leroy, F. and DeVuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science and Technology, 15,* 67–78.

Leroy, F., Verluyten, J. and De Vuyst, L. (2006). Functional meat starter cultures for improved sausage fermentation. *International. Journal of Food Microbiology, 106,* 270–285.

Lopandic, K., Zelger, S., Banszky, L. K., Eliskases-Lechner, F. and Prillinger H. (2006). Identification of yeasts associated with milk products using traditional and molecular techniques. *Food Microbiology*, *23*, 341-350.

Lore, T. A., Mbugua, S. K. and Wangoh, J. (2005). Enumeration and identification of microflora in suusa, a Kenyan traditional fermented camel milk product. *LWT-Food Science and Technology, 38(2),* 125-130.

Mainville, I., Robert, N., Lee, B. and Farnworth, E. R. (2006). Polyphasic characterization of the lactic acid bacteria in kefir. *Systematic and Applied Microbiology, 29,* 59-68.

Maphill map gallery. Available at; http://www.maphill.com/search results/?search=kiisii%20 county. Accessed on 25 September 2019.

Marshal, V. M. (1986). The microflora and production of fermented milks. *Progress in Industrial Microbiology, 23,* 1–44.

Marshall, E., and Mejia, D. (2012). Food and Agriculture Organisation Bulletin, Rome, Italy.

Marshall, V. M. E. and Law, B. A. (1984). The physiology and growth of dairy lactic acid bacteria. *Advances in the Microbiology and Biochemistry of Cheese and Fermented milk, 12*, 67-98.

Mathara, J. M., Schillinger, U., Kutima, P. M., Mbugua, S. K. and Holzapfel, W. H. (2008). Functional properties of Lactobacillus plantarum strains isolated from Maasai traditional fermented milk products in Kenya. *Current Microbiology, 56,* 315 – 321.

Mathara, J. M. (1999). *Studies on lactic acid producing microflora in mursik and kule naoto, traditional fermented milks from Nandi and Masai communities in Kenya* (Doctoral dissertation, University of Nairobi).

Mathara, J. M., Schillinger, U., Kutima, P. M., Mbugua, S. K. and Holzapfel, W. H. (2004). Isolation, identification and characterisation of the dominant microorganisms of kule naoto: the Maasai traditional fermented milk in Kenya. *International Journal of Food Microbiology, 94*(3), 269-278.

McCartney, A. (2002). Application of molecular biological methods for studying probiotics and the gut flora. *British. Journal of Nutrition. 88,*29–37.

Mengkol, T. (2011). Biotherapy for and protection against gastrointestinal pathogenic infections via action of probiotic bacteria; Review. *International Journal of Science and Technology, 5(01),* 108 - 128.

Miller, H. G. and Singh, V. (1994). Potential field tilt—a new concept for location of potential field sources. *Journal of Applied Geophysics, 32(2),* 213-217.

Mitall, B. K. and Garg, S. K. (1995). Anticarcinogenic, hypocholesterolemic, and antagonistic activities of *Lactobacillus acidophilus*. *Critical Reviews in Microbiology, 21*(3), 175-214.

Mokua, R. A. (2004). *Effect of Kenyan fermented milk on Escherichia coli.* (Master of Science), The Graduate College University of Wisconsin-Stout Menomonie, USA.

Moreno, M.F., P. Sarantinopoulos, E. Tsakalidou. and L. De Vuyst, (2006). The role and application of enterococci in food and health. *International journal of food microbiology, 106,* 1-24

Motarjemi, Y. and Nout M. R. J. (1995). Food fermentation safety and nutritional assessment. *Bulletin of World Health Organization, 74,* 553–558.

Mugula, J. K., Nnko, S. A. M. and Sørhaug, T. (2001). Changes in quality attributes during storage of togwa, a lactic acid fermented gruel. *Journal of Food Safety*, *21*(3), 181-194.

Muigei, S. C., Shitandi, A., Muliro, P. and Bitonga, O. R. (2013*).* Production of Exopolysaccharides in the Kenyan Fermented Milk, Mursik. *International Journal of Science and Research, 2 (12),* 2319-7064.

Muinde, A. (2011). *Popular milk, mursik now in shops.* Retrieved on December 12, 2012, from Farmbiz Africa: http://farmbizafrica.com/index.php

Munshi, A. (2012). *DNA Sequencing Methods and applications.* Punjab: Central University of Punjab, pp 67-89.

Muyzer, G. de Waal E. and Uitterlinden, A. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied Environmental Microbiology, 59,* 695–700.

Muyzer, G., Brinkhoff, T., Nubel, U., Santegoeds, C., Schafer, H. and Wawer, C. (2004). *Denaturing gradient gel electro-phoresis (DGGE) in microbial ecology*. In: Kowalchuk G.A., de Bruijn F.J., Head I.M., Akkermans A.D.L., van Elsas J.D. (eds): Molecular Microbial Ecology Manual. Kluwer Academic Publishers Dordrecht, pp 743–769.

Narvus, J. A. and Gadaga, T. H. (2003). The role of interaction between yeasts and lactic acid bacteria in African fermented milks, A reiew. *International Journal of Food Microbiology, 86,* 51-60.

Nduko, J. M., Matofari, J. W., Nandi, Z. O. and Sichangi, M. B. (2017). Spontaneously fermented kenyan milk products: A review of the current state and future perspectives. *African Journal of Food Science, 11(1),* 35-36

Neti, Y., Erlinda, I. D. and Virgilio, V. G. (2011). The effect of spontaneous fermentation on the volatile flavor constituents of durian. *International Food Research Journal, 18*(2), 5-6.

Nieminen, M. T, Novak-Frazer, L, Collins, R, Dawsey, S.P, Dawsey, S.M, Abnet, C.C., White, R. E, Freedman N.D, Mwachiro, M., Bowyer, P. and Salaspuro, M. (2013). Alcohol and acetaldehyde in African fermented milk Mursik–A possible etiologic factor for high incidence of oesophageal cancer in Western Kenya. *Cancer Epidemiology Biomarch. 22(1),* 69-75.

Njage, P. M. K., Dolci, S., Jans, C., Wangoh, J., Lacroix, C. and Meile, L. (2011). Characterization of yeasts associated with camel milk using phenotypic and molecular identification techniques. *Research Journal of Microbiology*, *6*(9), 678.

Nout, M. J. (1981). *Aspects of the manufacture and consumption of Kenyan traditional fermented beverages* (Doctoral dissertation, Nout), Wageningen University, Netherlands.

Nyambane, B., Thari, M., Wangoh, W., Njage J. and Patrick, M. (2014). Lactic acid bacteria and yeasts involved in the fermentation of *amabere amaruranu,* a Kenyan fermented milk. *Food Science and Nutrition,* 2*(6),* 692–699.

O'Connor, C.B., Mezgebu, S. and Zewd, Z. (1993). *Improving the efficiency of butter making in Ethiopia.* FAO *World Animal Reviews, 6,* 50–53.

Obodai, M. and. Dodd, C. E. R. (2006). Characterization of dominant microbiota of a Ghanaian fermented milk product, nyarmie, by culture- and non-culture-based methods. *Journal of Applied Microbiology, 100(6),* 355–1363.

Ostad, S. N., Salarian, A. A., Ghahramani, M. H., Fazeli, M. R., Samadi, N. and Jamalifar, H. (2009). Live and heat-inactivated lactobacilli from faeces inhibit *Salmonella typhi* and *Escherichia coli* adherence to Caco-2 cells. *Folia Microbiology*, *54,* 157-160.

Ouadghiri, M., Amar, M., Vancanneyt, M. and Swings, J. (2005). Biodiversity of lactic acid bacteria in Moroccan soft white cheese (Jben). M*icrobiology Letters, 251*(2), 267-271.

Owusu-Kwarteng, J. F., Akabanda, J. F., Nielsen, D. S. K. Tano-Debrah, K. Glover, R. L.K. and Jespersen, L. (2012). Identification of lactic acid bacteria isolated during traditional fura processing in Ghana *Food Microbiology*, *32(1),*72–78.

Park, S., Ji, Y. and Parketal, H. (2016). Evaluation of functional properties of lactobacilli isolated from Korean white kimchi, *Food Control*, *69*, 5–12.

Parkes, G. C., Sanderson, J. D. and Whelan, K. (2009). The mechanisms and efficacy of probiotics in the prevention of Clostridium difficile-associated diarrhoea. *Lancet Infectious Diseases*, *9,* 237-244.

Parrez, S., Malik, K. A., AhKang, S. and Kim, H. Y. (2006). Probiotics and their fermented food products are beneficial for health. *Journal of Applied Microbiology*, *100,*1171 - 1185.

Patel, K., Wakhisi, J. Mining S., Mwangi, A. and Patel, R. (2013). Oesophageal cancer, the topmost cancer at MTRH in the rift valley, Kenya, and its potential risk factors. *Oncology 2013,* 1-9.

Pederson, C. S. (1971). Microbiology of food fermentations. *Microbiology of Food Fermentations; 23(2*),1-48.

Pereira-Dias, S., Potes, M. E., Marinho, A., Malfeito-Ferreira, M. and Loureiro, V. (2000). Characterisation of yeast flora isolated from an artisanal portuguese ewes cheese. *International Journal of Food Microbiology*, *60*, 55-63

Pereira-Dias, S., Potes, M. E., Marinho, A., Malfeito-Ferreira, M. and Loureiro, V. (2000). Characterisation of yeast flora isolated from an artisanal Portuguese ewes' cheese. *International Journal of Food Microbiology*, *60*(1), 55-63.

Prema, P., Bharathy, S., Palavesa, A, Sivasubramanian, M. and Immanuel, G. (2006). Detection, purification and efficacy of warnerin produced by *Staphylococcus warneri. World Journal of Microbiology and Biotechnology, 22* (8), 865-872.

Rahman, I. E. A., Dirar, H. A. and Osman, M. A. (2009). Microbiological and biochemical changes and sensory evaluation of camel milk fermented by selected bacterial starter cultures. *African Journal of Food Science*, *3*(12), 398-405.

Randazzo, C.L., Torriani, S., Akkermans, A.D.L., de Vos, W. M. and Vaughan, E. E. (2002). Diversity, dynamics, and activity of bacterial communities during production of an artisanal sicilian cheese as evaluated by 16S rRNA analysis. *Applied Environmenta Microbiology, 68,* 1882–1892.

Rattanachaikunsopon, P. and Phumkhachorn, P. (2010). Lactic acid bacteria: their antimicrobial compounds and their uses in food production. *Annual Biological Ressearch*, *1*(4), 218-228.

Ray, R.C. and Joshi V.K. (2014). Fermented Foods: Past, present and future scenario, in *Microorganisms and Fermentation of Traditional Foods*, R.C. Rayand, D. Montet, Eds, pp.1–36, CRC Press, Boca Raton, Fla, USA.

Reddy, G. V., Friend, B. A., Shahani, K. M. and Farmer, R. E. (1983). Antitumor activity of yogurt components. *Journal of Food Protection*, *46*(1), 8-11.

Romano, P., Ricciardi A., Salzano, G. and Suzzzi G. (2001). Yeasts from water buffalo Mozzarella, a traditional cheese of the Mediterranean area. *International Journal of Food Microbiology*, *69,* 45-51.

Sakurai M., Suzuki K., Onodera M., Shinano T. and Osaki M. (2007). Analysis of bacterial communities in soil by PCR-DGGE targeting protease genes. *Soil Biology and Biochemistry*, *39,* 2777–2784.

Salminen, S. and Von Wright, A. (2004). *Lactic acid bacteria: microbiological and functional aspects*. CRC Press, pp 23-34.

Samelis, J., Maurogenakis, F. and Metaxopoulos, J. (1994). Characterisation of lactic acid bacteria isolated from naturally fermented Greek dry salami. *International Journal of Food Microbiology, 23(2),* 179-196.

Sanders, M. and Veld, J. (1999). Bringing a probiotic-containing functional food to the market: microbiological, product, regulatory and labelling issues. *Antonie van Leeuwenhoek, 76,* 293–315.

Sansonnetti, P. J. (2008). Host-bacteria homeostasis in the healthy and inflamed gut. *Current Opinion of Gastroenterology, 24,* 435 - 439.

Sarantinopoulos, P., Andrighetto, C., Georgalaki, M. D., Rea, M. C., Lombardi, A., Cogan, T. M., ...and Tsakalidou, E. (2001). Biochemical properties of enterococci relevant to their technological performance. *International Dairy Journal*, *11*(8), 621-647.

Satish, R., Ragu, V., Kanmani, P., Yuvaraj, N., Paari, K., Pattukumar V. and Arul, V. (2011). Isolation, Characterization and Identification of a Potential Probiont from South Indian Fermented Foods (*Kallappam*, *Koozh* and *Mor Kuzhambu*) and Its Use as Biopreservative. *Letters in Applied Microbiology, 53* (4), 481–487.

Savadogo, A., Cheik, A.T., Ouattara, I., Bassole, H. N. and Traore, A. S. (2004). Microorganisms involved in Fulani traditional fermented milk in Burkina Faso. *Pakistan journal of Nutrition*, *3,* 134 – 139.

Schillinger, U. and Lücke, F. K. (1989). Antibacterial activity of Lactobacillus sake isolated from Harrigan, W. F., & McCance, M. E. (1976). *Laboratory methods in food and dairy microbiology*. Academic Press Inc. (London) Ltd. Meat. *Applied Environmental Microbiology*, *55*(8), 1901-1906.

Seeley, T. D. (2009). *The wisdom of the hive: the social physiology of honey bee colonies*. Harvard University Press, pp 567-670.

Simova, E., Beshkova, D., Angelov, A., Hristozova, T., Frengova, G. and Spasov, Z. (2002*).* Lactic acid bacteria and yeasts in kefir grains and kefir made from them. *Journal of Industrial Microbiology and Biotechnology, 28,* 1-6.

Sneath, P. H., Mair, N. S., Sharpe, M. E. and Holt, J. G. (1986). *Bergey's manual of systematic bacteriology. Volume 2*. Williams and Wilkins, pp 89-102.

Soda, M., N. Ahmed., N. Omran, G. Osman. and A. Morsi. (2017). Isolation, Identification and Selection of Lactic Acid Bacteria Cultures for Cheesemaking. *Emirates Journal of Food and Agriculture, 15*(2), 51-71,

Staley, J.T. (2006). The bacterial species dilemma and the genomic-phylogenetic species concept. Philos. Trans. R. Soc. *London. Biological. Science, 361,* 1899–1909.

Stepanenko, P. P. (1999). Mikrobiologiya moloka i molochnykh produktov (Microbiology of Milk and Milk Products). *Sergiev Posad: Vse dlya Vas—Podmoskov'e, 5(*2), 110-114.

Stiles, M.E. and Holzapfel, W.H. (1997). Lactic acid bacteria of foods and their current taxonomy. *International Journal of. Food Microbiology., 36,* 1–29.

Sudhanshu, S. Behera, Ramesh, C. Ray. and Nevijo Zdolec. (2018). *Lactobacillus plantarum* with Functional Properties: An Approach to Increase Safety and Shelf-Life of Fermented Foods. *Biomedical Research International, 7,* 1-50.

Surajit, Das. (2014). Understanding molecular identification and polyphasic taxonomic approaches for genetic relatedness and phylogenetic relationships of microorganisms. *Journal of Microbiological Methods,103,* 80-100

Suzzi, G., Schirone M., Martuscelli M., Gatti M., Fornasari, M. E. and Neiani E. (2003). Yeasts associated with Menteca. *FEMS Yeast Research, 3,* 159 – 166.

Tambekar, D. H. and Bhutada, S. A. (2010). An evaluation of probiotic potential of Lactobacillus sp. from milk of domestic animals and commercially available probiotic preparations in prevention of enteric bacterial infections. *Recent Research in Science and Technology, 2(10),* 67-69

Tamine, A.Y. and Robinson, R. K. (1988). Fermented milks and their future trends: technological aspects. *Journal of Dairy Research, 55,* 281-307.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, *24*(8), 1596-1599.

Todorov, S. D., LeBlanc, J. G. and Franco, B. D. (2012). Evaluation of the probiotic potential and effect of encapsulation on survival for Lactobacillus plantarum ST16Pa isolated from papaya. *World Journal of Microbiology and Biotechnology, 28*(3), 973-984.

Ukeyima, M. T., Enujiugha, V. N. and Sanni, T. A. (2010). Current applications of probiotic foods in Africa. *African Journal of Biotechnology, 9*(4), 25-26.

Vanderpool, C., Yan, F. and Polk, D. B. (2008). Mechanisms of probiotic action: Implications for therapeutic applications in inflammatory bowel diseases. *Inflammatory Bowel Disease*, *14*, 1585 - 1596.

Varnam, A.H. (1993). *The exploitation of microorganisms in the processing of dairy products*. In Jones, D.G. (ed.) Exploitation of microorganisms. Chapman and Hall, London. pp. 273-296.

Walter, J., Hertel, C., Tannock, G.W., Lis, C.M., Munro, K. and Hammes, W.P. (2001). Detection of Lactobacillus, *Pediococcus, Leuconostoc,* and Weissella species in human faeces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology, 67,* 2578-2585.

Wang, B., Li J., Li Q., Zhang, H. and Li, N. (2009). Isolation of adhesive strains and evaluation of the colonization and immune response by *Lactobacillus plantarum* L2 in the rat gastrointestinal tract. *International Journal of Food Microbiology, 132,* 59 - 66.

Wartiainen, I., Eriksson, T., Zheng, W. and Rasmussen, U. (2008). Variation in the active diazotrophic com-munity in rice paddy-nifH PCR-DGGE analysis of rhizosphere and bulk soil. *Applied Soil Ecology, 39,* 65–75.

Wessels, S., Axelsson, L., Hansen, E. B. and De Vuyst, L. (2004). The lactic acid bacteria, the food chain, and their regulation. *Trends in Food Science and Technology, 15*, 498–505.

Yahuza, M.L. (2001). *Small - holder dairy production and marketing constrains in Nigeria*. In: Rangnekegr D, Thorpe W (Eds). Proceedings of a South – South workshop held at National Dairy Development Board (NDDB). Anand, India, 13 – 16 March 2001, and ILRI (International Livestock Research Institute), Nairobi, Kenya.

Yelnetty, A., Purnomo, H. and Mirah, A. (2014). Biochemical characteristics of lactic acid bacteria with proteolytic activity and capability as starter culture isolated from spontaneous fermented local goat milk. *Journal of Natural Sciences Research*, *4*(10), 137-146.

APPENDICES

APPENDIX 1: Initial Log results of microbial counts (Coliforms, Total viable counts, Lactic acid bacteria counts, Yeasts and Moulds counts);

SOURCE	SAMPLE	REP	LOGCOLI	LOGTVC	LOGLABS	LogYM
Commercial	A15	1	6.96	7.83	7.59	7.17
Commercial	A15	2	6.90	7.86	7.58	7.18
Commercial	A15	3	6.95	7.84	7.60	7.18
Commercial	A11	1	6.04	7.42	6.94	6.72
Commercial	A11	2	6.27	7.43	6.99	6.63
Commercial	A11	3	6.19	7.45	7.04	6.65
Commercial	A18	1	6.55	7.84	7.25	6.71
Commercial	A18	2	6.55	7.83	7.20	6.63
Commercial	A18	3	6.53	7.83	7.23	6.65
Commercial	A9	1	6.54	7.50	6.69	6.67
Commercial	A9	2	6.47	7.47	6.67	6.60
Commercial	A9	3	6.55	7.49	6.65	6.71
Commercial	A24	1	6.72	7.08	4.22	6.68
Commercial	A24	2	6.77	7.06	4.22	6.60
Commercial	A24	3	6.77	7.04	4.30	6.68
Commercial	A22	1	6.94	8.05	7.17	6.63
Commercial	A22	2	6.89	8.03	7.11	6.65
Commercial	A22	3	6.95	8.04	7.19	6.58
Commercial	A21	1	5.78	6.84	7.14	5.68
Commercial	A21	2	5.73	6.85	7.10	5.67
Commercial	A21	3	5.30	6.82	7.08	5.89
Commercial	A16	1	5.94	6.87	7.83	7.61
Commercial	A16	2	5.67	6.92	7.82	7.60
Commercial	A16	3	5.67	6.90	7.84	7.60
Commercial	A13	1	6.54	8.05	7.38	7.38
Commercial	A13	2	6.49	8.04	7.35	7.36
Commercial	A13	3	6.40	8.03	7.39	7.36
Commercial	A26	1	7.24	8.05	6.99	6.98

Commercial	A26	2	7.24	8.04	6.94	7.02
Commercial	A26	3	7.26	8.03	6.97	7.00
Commercial	A17	1	5.93	8.05	7.63	6.86
Commercial	A17	2	5.73	8.04	7.62	6.92
Commercial	A17	3	5.94	8.04	7.63	6.89
Commercial	A14	1	4.88	7.12	7.75	6.75
Commercial	A14	2	4.87	7.14	7.73	6.74
Commercial	A14	3	5.58	7.14	7.74	6.69
Commercial	A7	1	7.34	7.26	8.00	7.23
Commercial	A7	2	7.35	7.29	8.00	7.25
Commercial	A7	3	7.37	7.28	8.00	7.24
Commercial	A6	1	6.55	5.57	7.83	6.58
Commercial	A6	2	6.63	5.57	7.83	6.51
Commercial	A6	3	6.66	5.53	7.84	6.52
Commercial	A10	1	7.02	7.42	7.23	6.68
Commercial	A10	2	6.94	7.39	7.23	6.62
Commercial	A10	3	6.97	7.41	7.23	6.62
Commercial	A12	1	7.32	7.59	8.05	7.22
Commercial	A12	2	7.29	7.58	8.04	7.17
Commercial	A12	3	7.30	7.59	8.05	7.20
Commercial	A4	1	7.01	7.33	7.48	7.07
Commercial	A4	2	6.94	7.34	7.48	7.04
Commercial	A4	3	6.97	7.35	7.49	7.01
Commercial	A1	1	5.72	7.19	7.59	7.23
Commercial	A1	2	5.18	7.16	7.57	7.23
Commercial	A1	3	5.62	7.17	7.59	7.26
Commercial	A8	1	5.65	3.82	7.29	6.69
Commercial	A8	2	4.92	4.60	7.27	6.72
Commercial	A8	3	5.90	5.57	7.28	6.75
Commercial	A3	1	3.82	7.21	7.27	7.09
Commercial	A3	2	3.52	7.23	7.24	7.10
Commercial	A3	3	3.82	7.24	7.30	7.51
Commercial	A25	1	6.40	4.12	5.80	7.01
Commercial	A25	2	6.45	5.54	5.98	7.00

Commercial	A25	3	6.50	5.58	6.00	7.02
Commercial	A5	1	3.82	7.00	7.19	6.48
Commercial	A5	2	4.64	6.97	7.15	6.41
Commercial	A5	3	5.57	7.01	7.12	6.47
Commercial	A20	1	6.36	5.87	6.97	6.69
Commercial	A20	2	6.48	6.05	6.94	6.65
Commercial	A20	3	6.47	6.05	6.94	6.67
Commercial	A19	1	6.27	4.48	7.50	6.75
Commercial	A19	2	6.18	4.82	7.47	6.71
Commercial	A19	3	6.21	5.59	7.48	6.77
Commercial	A23	1	6.55	6.06	5.70	7.12
Commercial	A23	2	6.47	6.07	5.92	7.08
Commercial	A23	3	6.52	6.06	5.67	7.09
Commercial	A27	1	3.82	6.20	7.33	6.40
Commercial	A27	2	4.60	6.20	7.34	6.42
Commercial	A27	3	5.54	6.30	7.34	6.36
Commercial	A28	1	4.67	6.60	7.33	6.82
Commercial	A28	2	4.88	6.66	7.35	6.69
Commercial	A28	3	5.58	6.69	7.33	6.78
Commercial	A29	1	7.01	7.79	5.53	6.48
Commercial	A29	2	7.08	7.79	5.85	6.52
Commercial	A29	3	7.07	7.78	5.58	6.48
Commercial	A30	1	7.66	7.51	5.70	6.52
Commercial	A30	2	7.67	7.52	5.90	6.57
Commercial	A30	3	7.66	7.51	5.66	6.55
Commercial	A31	1	7.29	7.26	3.52	5.77
Commercial	A31	2	7.26	7.25	5.57	5.74
Commercial	A31	3	7.28	7.26	5.53	5.76
Commercial	A32	1	7.36	7.23	5.57	5.73
Commercial	A32	2	7.32	7.15	5.83	5.95
Commercial	A32	3	7.32	7.20	5.57	5.76
Commercial	A33	1	7.47	7.29	3.52	0.00
Commercial	A33	2	7.44	7.31	3.52	0.00
Commercial	A33	3	7.47	7.30	3.52	0.00

Commercial	A34	1	7.74	7.58	7.08	0.00
Commercial	A34	2	7.73	7.59	7.15	0.00
Commercial	A34	3	7.75	7.58	7.05	0.00
Commercial	A35	1	7.40	7.28	6.91	0.00
Commercial	A35	2	7.38	7.28	6.94	0.00
Commercial	A35	3	7.40	7.30	6.94	0.00
Commercial	A36	1	7.36	6.99	6.87	6.62
Commercial	A36	2	7.35	7.01	6.91	6.69
Commercial	A36	3	7.35	6.96	6.86	6.69
Commercial	A37	1	7.15	6.83	6.44	6.32
Commercial	A37	2	7.13	6.81	6.49	6.40
Commercial	A37	3	7.11	6.83	6.36	6.33
Commercial	A38	1	7.70	7.36	7.10	6.73
Commercial	A38	2	7.70	7.38	7.05	6.64
Commercial	A38	3	7.71	7.38	7.08	6.76
Commercial	A39	1	7.53	6.86	6.64	6.67
Commercial	A39	2	7.50	6.90	6.64	6.62
Commercial	A39	3	7.51	6.91	6.71	6.64
Household	1	1	8.93	9.77	10.85	7.41
Household	1	2	8.55	9.99	10.89	7.3
Household	1	3	8.74	9.88	10.87	7.36
Household	2	1	8.19	10.92	10.85	8.16
Household	2	2	8.26	10.98	11	8.16
Household	2	3	8.22	10.95	10.93	8.16
Household	3	1	8.16	10.96	11.04	8.08
Household	3	2	8.39	10.84	11.01	8.08
Household	3	3	8.23	10.9	11.03	8.08
Household	4	1	7.47	10.02	10.71	8.04
Household	4	2	6.92	10.24	10.6	7.95
Household	4	3	7.2	10.13	10.66	8
Household	5	1	7.23	10.14	10.96	8.22
Household	5	2	6.79	10.06	11.32	8.17
Household	5	3	7.01	10.1	11.14	8.2
Household	6	1	6.68	9.91	10.24	8

Household	6	2	6.73	9.8	10.51	8.16
Household	6	3	6.71	9.86	10.38	8.08
Household	7	1	6	9.15	9.77	7.98
Household	7	2	6.52	9.74	9.19	8.05
Household	7	3	6.26	9.45	9.48	8.02
Household	8	1	9.01	10.31	10.63	9.05
Household	8	2	9.01	10.44	10.93	8.64
Household	8	3	9.01	10.34	10.78	8.85

APPENDIX 2: Partial DNA sequences

Sample number 24 ($A^6$8) Lac1 DNA sequence

5'TTATKGGAGCACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTG
TTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAA
AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGT
CCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGC
CTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGG
ACAGTGGAACTCCATGTGTASGGGTGRAATCA**3'**

Sample number 24 ($A^6$8) Lac2 DNA sequence

5'ATCAAGTTTCCAGTTTCCGATGCACTTCTTCGGTTGAGCCGARGGCTTTCACATCA
GACTTAAAAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCC
ACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAAATA
CCGTCAATACCTGAACAGTTACTCTCAGATATGTTCTTCTTTAACAACAGAGTTTTAC
GAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGACTTTCGTCCATTGT
GGAAGATTCCCCTACTGCTA**3'**

Sample number 27 ($M^1$2W) Lac1 DNA sequence

5'GGKMYTARGARMAACSCCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCT
GTTGTTAAAGAAGAACAYMTYTKAGAGTAACTGTTCAGGTATTGACGGTATTTAAC
CAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC
GTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTG
AAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGA
AGAGGACAGTGGAACTCCATGTGTAGCGGTGRAATCA**3'**

Sample number 27 ($M^1$2W) Lac2 DNA sequence

5'CCTTKYKRGMMWMAGTTTCCAGTTTCCGAKGCACTTCTTSGGTKGAGCCGARGGC
TTTCACATCAGACTTAAAAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGAC
AACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTC
TGGTTAAATACCGTCAATRCCTGAACAGTTACTCTCAGATATGTTCTTCTTTAACAAC
AGAGTTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGACTTT
CGTCCATTGTGGAAGATTCCCTACTGCTA**3'**

Sample number 28 ($B^6$2W) Lac1 DNA sequence

5'CRGGRRSCAMSCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATCA
GGGAAGAACAAMYGTGTAAGTAACTGTGCACRTCTTGACGGTACCTGATCAGAAAG
CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCC
GGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCC
ACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAA
AGTGGAATTCCATGTGTAGCGGTGRAATCA**3'**

Sample number 28 ($B^6$2W) Lac2 DNA sequence

5'GWWCKMCTCAAGTTTTCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTT
TCACATCAGACTTAAAAAACCGCCTACGCGCGCTTTACGCCCAATAATTCCGGATAA
CGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTG
ATCAGGTACCGTCAAGAYGTGCACAGTTACTTACACRTTTGTTCTTCCCTGATAACA
GAGTTTTACGATCCGAAGACCTTCATCACTCACGCGGCGTTGCTCCGTCAGGCTTTC
GCCCATTGTGGAAGATTCCCTACTGCTA**3'**

Sample number 6 (R^3SC) ITS-1 DNA sequence

5'GMMAARSGRGKKGKAAACYTTCGGTCTGCTACCTCTTACCCATGTCTTTTGAGTAC
CTTCGTTTCCTCGGCGGGTCCGCYCGCCGATTGGACAACATTCAAACCCTTTGCAGT
TGCAATCAGCGTCTGAAAAAACATAATAGTTACAACTTTCAACAACGGATCTCTTGG
TTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAAT
TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCA
TGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTC
GCCTCCGCGTGTAGACTCGCCTTAAAACAATTGGCAGCCGGCGTATTGATTTCGGAG
CGCAGTACATCTCGCGCTTTGCACTCATAACGACGACGTCCAAAAGTACATTTTTAC
ACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCG
GAGGAA**3'**

Sample number 9 (B2SW) ITS-4 DNA sequence

5’AGGTCMRKKYCCTCGAGGTYATKRGTGTRMWAATGTACTTKTGGACGTCGTCRKT
ATGAGTGCAAAGYGCGAGATGTACTGCGCTCCGAAATCAATACGCCGGCTGCCAAT
TGTTTTAAGGCGAGTCTACACGCAGAGGCGAGACAAACACCCAACACCAAGCAGAG
CTTGAAGGTACAAATGACGCTCGAACAGGCATGCCCCATGGAATACCAAGGGGCGC
AATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTACTTATCG
CATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGT
AACTATTTATGTTTTTTCAGACGCTGATTGCAACTGCAAAGGGTTTGAATGTTGTCCA
ATCGGCGGGCGGACCCGCCGAGGAAACGAAGGTACTCAAAAGACATGGGTAAGAG
GTAGCAGACCGAAGTCTACAAACTCTAGGTAATGATCCTTCCGCAGGTTCACCTACG
GA**3’**

Sample number 9 (B2SW) ITS-1 DNA sequence

5’ACGGGAAGACTTCCGKAGGTGCTACCTGYKRCCCATGWYTTYTGAGKCCTWCGAT
TCCTCGGCGGGTCCGCCCGCCGATTGGACAACATTCAAACCCTTTGCAGTTGCAATC
AGCGTCTGAAAAAACATAAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTG
AATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTG
TTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCCTCT
GCGTGTAGACTCGCCTTAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGT
ACATCTCGCGCTTTGCACTCATAACGACGACGTCCAAAAGTACATTTTTACACTCTT
GACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGRA
A**3’**

Sample number 24 (A^{6}8) 907-R DNA sequence

5’CMRGCCGGGWGTGCCTTAYGCGTTAGCTGCRACACTGAAKRASTRRAWAYACCT
MCCATCTAACACACTCATCKTTACCGCGTGGACTACCCAGGGATCTAATCCTGGTTG
CTCCCCAACCTTTCCSGCCTCARSGGYMRKTAACAGACAAGATGCCCSCCTCCCCAC
CTGGGGTCTTTCCATAATTTACCAATTTCACCTCTACCCTGGGAAATCCCCCTCCCTC
CTTCACACTCTARKYTGCCCRKATYMMATGGACCTTCCCSGGTAGASCCGGGGATTT

CACATTTRAATGTAMRAACCCSCCAGCGCTCCTTTACSSCCCAWYATTTCCAGCAAA
SSTTKGCCCCTTACKATTACCCGSGSTGGTGGSACSAAAKTARSCCGGGSTTTTTTGGY
KGGAACCGKYMTCGGGATGACCGSCKAATCTGATTTTTGTTCTTAAAACCTTCTTCA
CACACACGAYATTGCTGGATTCTGGWTTCCCCCGTTGTCCAATATTCCCCACTGCTG
CCTTTGCCGGAGTCTCCCTACTGCTGCCTCCCCAGTGAGGCTGATCATCCTCTCAGAC
CARMTGTGGATSATCRCCCTGTCAGGYCATTAATCCATCRWGRMCTTGATGARACG
YWAGCTCCTCMACTAGCTAMTGGMSCCTTTGACCMTMMRGYGKCATGMSGYATTA
GCTCCTTTTTCATARAAACATTGCCCACCCATGGATAGTATCCTATKAGYWACTGTT
TCGWMGKGCACTAMSGTCSAGATGGGCRKGYKACTTGCRTGTGACTCRCMTGYYCS
CMRCTCTCGTTTTKASSGTGWAMMAAARMTCTAGTGGAAWAARGAAGCGTWCGAC
TYGCATGTATTAGGCACGCCGMCASCGTTCGTCCTGAKMCATGATTYCAAACTTYT**3**
’

Sample number 24 (A^{6}8) 1492-R DNA sequence

5’CMWGWAGGGCGGGGTGTGCTCCACTATGYKRSYTCRMYSACKTGCTKRWGGTM
MAAMCTMCTCCCATGGTGYGACGGGCGGGTGWACGSCCCCGAACAAATTATTCGC
GSGTGTGGTGATCCGATTTTACTAGATATTCCMCTTTCGTGSGCTAGATGTGCACAG
YGMAMTCAA+ATCTGAGAAGGTTTTTAAAAATAASTTAGATKTCSSAATTTASCTTC
CCACTGACTTCCCCTTTGAASMASKGGGGWASCCCAGGGMATAAGGGCCATGAGGA
CTTGACKYMTTCCCCACCTTCCTCCGGTTTGYCACCGGMRKYYTSTCTAAAGGGCCC
ACCCAAASATGSTGRCWAMTAAWRATGGGGGYTGCSCTCGYTGGAGTAATTAACCC
AACATCTCACRACACGAGATGACRACCRTCATGCACCTGCTGYTTTGYCCGCCCCTG
GGAAGAAATATCCWTCTATGGATACAASGAACCCATAAMCTGCCTGGGAAGGTTCT
GCGGGTTGCTTWTAAATAAACCGCATGATCCACCGKTSGGGCGGGCCCCCATTAATT
CCTTTGASWTTCTTGCTTGCGGACTTACTCCGCAGGSKGYGTGATTAAYKMGYTGCC
TGMSACACTGAAKAACTAACTTACCCAACATCTASCACACATCTTATTACAGSGKGR
ACTACCAGGGTATCTAATCCTGTGYTGCTCCCCCACTTTTTCGCCGCCTCAKCGTYW
AYTATRARMCARAGYCGCCTTCTTCRCCACCGGTGTTCTTCMYAATATCTACATAWT
YWYCRCCTCTACACTGARTAYWCCACWMCTCTTCTCTCACACTMTAKTCTGCAYKT
MTMAWGATGCASCTCCSAKTGAKAMGSSSSGGGTTATTTCACATCTKWMTGWAMA
MMGCCGYGCTACWCGCTCTTYTACGCMCWARWYWYTCSASARMAACTTSTCGMC
CCYWCTTTMRTTATTASCGCGTGCTGSTGRCGACARAWKWTMSGCGGGKTCTTYTG
TCTAGAAKRACTASCGWCRKSATGCAATCMGCTGACYSAWMAGTSCTTGTWYATCY
GTAAAMACATGCAGTTCWYTACCRTCGTATRGCTTGCATCACGTCTGCCGCGATGCT
CGATMATACTACTGCATCGTCGATAGTACTCTAGCGTGCCGTGCCTCYCCAGTACCG
AGTTTGTGGG3**’.**

APPENDIX 3: Nacosti Research Permit

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: 140999

Date of Issue: 28/October/2019

RESEARCH LICENSE

This is to Certify that Mr.: MOSES SICHANGI of Egerton University, has been licensed to conduct research in Kisii on the topic: MOLECULAR CHARACTERIZATION OF THE MICROBIAL COMMUNITIES AND DEVELOPMENT OF STARTER CULTURE FROM TRADITIONALSPONTANEOUSLY FERMENTED MILK, Amabere Amarurasu IN KENYA. for the period ending : 28/October/2020.

License No: NACOSTI/P/19/2492

140999

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

APPENDIX 4: Research output

academicJournals

Vol. 11(1) pp. 1-11, January 2017
DOI: 10.5897/AJFS2016.1516
Article Number: E3C45B662071
ISSN 1996-0794

http://www.academicjournals.org/AJFS

African Journal of Food Science

Review

Spontaneously fermented kenyan milk products: A review of the current state and future perspectives

John Masani Nduko*, Joseph W. Matofari, Zacchaeus Okoth Nandi and Moses Barasa Sichangi

Department of Dairy and Food Science and Technology, Egerton University, P. O. Box 536-20115, Egerton, Kenya.

Received 15 September, 2016; Accepted 10 October, 2016

Many spontaneously fermented milk products are produced in Kenya, where they are integral to human diet and play a central role in enhancing food security and income generation. Some of these products have demonstrated therapeutic and probiotic effects although recent reports have linked some to death, biotoxin infections, and esophageal cancer. These products are mostly processed from poor quality raw materials under unhygienic conditions resulting to inconsistent product quality and limited shelf-lives. Though very popular, research on their processing technologies is low. This review provides a comprehensive summary of the most common spontaneously fermented milk products from Kenya including *Mursik*, *Kule naoto*, *Amabere amaruranu* and *Suusa*. Their production challenges and future perspectives are highlighted; emphasizing the need for application of high throughput biotechnologies in their study. Available literature on their microbiology, biochemistry, and chemical composition is summarized. Moreover, knowledge on the value of clean starting raw material, fermentation parameters definition, and employment of standard equipment are discussed.

Key words: Starter culture, probiotics, lactic acid bacteria, fermented milk, high throughput biotechnology, spontaneous fermentation, *Kule naoto*, *Mursik*, *Amabere amaruranu*